HARBOUR SURVEY AND GENETIC ANALYSIS OF NON-INDIGENOUS ASCIDIAN TUNICATES IN NEWFOUNDLAND

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by

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Abstract

This study is the first assessment of non-indigenous ascidians (NIA) in Newfoundland. Field work was conducted from 2006-2007 in four harbours to assess the abundance and biodiversity of megainvertebrates on wharf pilings, including indigenous and non-indigenous ascidians. Quadrat samples, visual surveys and photographic records were taken in each harbour. The most common species found in the survey were *Mytilus* spp. Two NIA were also found, *Botrylloides violaceus* and *Botryllus schlosseri.*

Variation in cytochrome oxidase I gene of mitochondrial DNA was analyzed for these NIA, as well as for two indigenous ascidians (*Boltenia echinata* & *Halocynthia pyriformis*), in order to determine within and between species variation for future use in genetic marker design and to identify probable source populations. There was less nucleotide dissimilarity within species (≤15.6 %) than among species (17.7 – 25.8 %). The probable source populations of *B. schlosseri* in Newfoundland are from locations in the Northwestern Atlantic and Europe, specifically the Atlantic and Mediterranean Sea coasts of France and Spain.

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List of Abbreviations

AIS	Aquatic Invasive Species
COI	Cytochrome *c* Oxidase I
DNA	Deoxyribonucleic acid
MED	Mediterranean
mtDNA	Mitochondrial DNA
NEA	Northeastern Atlantic
NIA	Non-indigenous ascidians
NIS	Non-indigenous species
NL	Newfoundland
nMDS	Non-metric multidimensional scaling
NWA	Northwestern Atlantic
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

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CHAPTER 1: INTRODUCTION

1.1 MARINE BIOINVASIONS AND TRANSPORT VECTORS

Bioinvasions are one of the leading marine environmental issues in the world and represent a serious global threat (Ruiz et al. 2000; Stachowicz et al. 2002; Occhipinti-Ambrogi and Galil 2004; Campbell et al. 2007). Introductions of non-indigenous marine organisms have been detected in all oceans of the world (Campbell et al. 2007). Non-indigenous marine species can threaten marine biodiversity, native species, the economy and human health (Blum et al. 2007).

Non-indigenous species (NIS) can be transported via several vectors. They are transported by vessels at the national, international and local levels. They may be attached to hulls or suspended in ballast water (Lambert and Lambert 1998; Lutzen 1999). Other potential transportation vectors for NIS are through the aquarium and aquaculture trades. NIS are particularly abundant in high risk ports that are characterized by high levels of shipping traffic. Once in these ports, aquatic invasive species (AIS) have the potential to spread by establishing themselves on wharves, other artificial structures, or in the natural environment.

1.2 GENERAL ASCIDIAN BIOLOGY

Ascidian tunicates (phylum Chordata, subphylum Tunicata, class Ascidiacea) are marine filter-feeding, hermaphroditic invertebrates that are found in all oceans (Plough

1978; Pollock 1998). Ascidians generally live at all depths, typically attached to both natural and human-made structures. For instance, they can be attached to other marine organisms (i.e. mussels, clams, etc.), to rocks and cliffs, and to manufactured structures such as wharf pilings, buoys, boats, and fishing and aquaculture equipment. Ascidians undergo indirect or mixed development, where they progress through egg, larval, tadpole, juvenile and adult stages. Ascidian larval and tadpole stages are relatively short in duration, typically spanning a few days or less, after which they settle onto a substrate and metamorphose. Therefore they are unable to disperse long distances and colonize new areas based upon current flow and larval drift (Locke et al. 2007). It is for this reason that shipping and other anthropogenic vectors are especially important in the spread of these organisms.

1.3 PROBLEMS WITH INVASIVE AND NON-INDIGENOUS ASCIDIANS

There are many ecological issues associated with invasive and non-indigenous ascidians. A species is considered invasive when it reaches extremely high population levels. The main concerns associated with ascidian invasive species are biofouling and decreased native species biodiversity (Karayucel 1997; Uribe and Etchepare 2002; Lambert and Lambert 1998, 2003; Carver et al. 2003; LeBlanc et al. 2007; Epelbaum et al. 2009). Non-indigenous ascidians are a significant biofouling problem for the aquaculture industry on both the east and west coasts of Canada. Over the past ten years, four ascidian tunicates have reached invasive levels at aquaculture sites in Nova Scotia

and Prince Edward Island, Canada. This major increase can lead to economic problems for the aquaculture industry. Impacts of invasive ascidians on the aquaculture industry include biofouling of the harvested species themselves as well as fouling of the growing and processing equipment. These impacts lead to decreased economic performance of the farms due to increased production costs. There are increased husbandry costs such as additional cleaning and processing, increased handling time and increased physical demands on the work crew (Carver et al. 2003; LeBlanc et al. 2003). In addition to processing costs, ascidians are threatening the mussel aquaculture industry by heavily biofouling mussel socks, causing decreased growth of mussels through competition for food and space by decreasing the water flow and food availability (Carver et al. 2003). A recent estimate of the economic impact of a single invasive ascidian species *Styela clava* on the Canadian aquaculture industry was 25% (i.e., $ 43,000,000) of a gross annual income of $ 170, 000, 000(Colautti et al. 2006). Ascidians have high rates of both sexual and asexual reproduction, and seasonally early recruitment rates relative to other benthic invertebrates (Arsenault et al. 2009; Ramsay et al. 2009). This allows them to colonize new substrata before other indigenous species such as bivalves have the chance to colonize an area following winter. Ascidians are also strong spatial competitors and once established can overgrow indigenous populations (Carver et al. 2003; Blum et al. 2006, Carver et al. 2006).

1.4 DESCRIPTION OF NON-INDIGENOUS AND INDIGENOUS ASCIDIAN SPECIES IN ATLANTIC CANADA

The non-indigenous ascidians that are causing problems in Atlantic Canada include *Styela clava*, *Ciona intestinalis*, *Botryllus schlosseri* and *Botrylloides violaceus*. *Styela clava* is a solitary, stalked ascidian (Carlton 1989; Lutzen 1999). Adults are about 15 cm long and 3 cm in diameter (Carver et al. 2006b). It is a competitive dominant that often occurs in dense stands in areas previously dominated by *Mytilus edulis* (Berman et al. 1992). *Styela clava* is originally an Asian species indigenous to the Sea of Japan and the coasts of Japan, Korea and China (Lutzen 1999). This invader was probably brought to the Atlantic coast of North America on ships from Europe sometime in the late 1600s (Berman et al. 1992; Carlton 1989). *C. intestinalis* is a solitary ascidian that has a gelatinous transparent tunic, similar in size to *S. clava,* and often co-occurs with other fouling ascidians. The native range of *C. intestinalis* is not clear but it is most likely a cosmopolitan species. It has been reported from the northeast Atlantic Ocean from the 1700-1800s (Plough 1978; Knott 1990; Carver et al. 2003, Carver et al. 2006b). *B. violaceus* is a colonial ascidian, native to Japan and the Pacific Northwest (Carver et al. 2003). Its zooids are approximately 2-4 mm long, forming thick encrusting mats that can be meters in length (Carver et al. 2006a). *B. schlosseri* is a colonial ascidian with zooids arranged in star-like arrays. Each zooid is about 1-2 mm long, and there are 5-20 zooids in a cluster (Carver et al. 2006a). *B. schlosseri* is a European species that is abundant along rocky shores of the Mediterranean and other European seas (Carver et al. 2006a; Paz et al. 2003). However, this invader is now found in many oceans worldwide.

There are several ascidian species that are indigenous to Newfoundland (Van Name 1945). Two of the most common are *Halocynthia pyriformis* and *Boltenia echinata*. *H. pyrifiormis* is a solitary ascidian that occasionally occurs in dense aggregations. It is red,

orange or peach in color and is 1-10 cm long. *B. echinata* is also a solitary ascidian, which is covered by cactus-like spines and has a brown-red surface with short siphons that are red. Both are northern boreal species that occur northward to the Arctic Ocean (Berrill 1950).

1.5 NEWFOUNDLAND'S RISK OF INVASION BY NON-INDIGENOUS ASCIDIANS AND EARLY DETECTION

Newfoundland is considered to be a high-risk area for the introduction of the non-indigenous species that have created problems elsewhere in Atlantic Canada (Therriault and Herborg 2008a; Therriault and Herborg 2008b). This concern is based on the fact that neighbouring provinces that have many shipping links with Newfoundland have already experienced severe problems from invasive non-indigenous ascidians (Locke et al. 2007; Therriault and Herborg 2008b). Several ports in Newfoundland have significant traffic with the Maritimes, including regular passenger and car ferries that traverse the Gulf of St. Lawrence from Port-aux-Basques and Argentia, Newfoundland and Labrador, to North Sydney, Nova Scotia. In addition to the ferries, there is regular local boat traffic among the Atlantic Provinces as well cargo vessels from Europe and other countries that frequently make port in Newfoundland.

Aquaculture is a growing industry in Newfoundland, with substantial export value. Given the significant impact that non-indigenous species have had in Atlantic Canada, it is important to know if and where these non-indigenous species are present in Newfoundland. At the start of this study, the presence of these ascidians was

undetermined. Therefore in 2006, a pilot survey of NIS was undertaken in four Newfoundland harbours that are particularly vulnerable to marine invasions, including Port-aux-Basques, Corner Brook, Botwood and Argentia. This project was a collaboration between Fisheries and Oceans Canada, Government of Newfoundland and Labrador, Memorial University of Newfoundland and the Newfoundland Aquaculture and Industry Association.

Although shipping vectors are the primary vector for the initial introductions of non-indigenous species, secondary spread of these species via small boats is also problematic and very difficult to prevent. Thus, eradication of these species once they have become established is generally an ineffective solution (Darling and Blum 2007). A more cost effective and realistic option is to support prevention of species introductions and the control of existing populations. Successful control of invasive organisms may be possible if they are detected early and if their distributions are known (Darling and Blum 2007).

1.6 MIGITATING THE EXPANSION OF INVASIVE AND NON-INDIGENOUS ASCIDIAN POPULATIONS

Mitigation measures have a much greater chance of success if invasions are detected early. One method of early detection is through genetic markers, in particular genetic markers. There are several genes that can be used in the development of markers for species detection. However, the gene that has been proposed to serve as the global bioidentification system in animals is the cytochrome *c* oxidase I gene (COI) (Hebert et al. 2003). The COI gene is located in the mitochondrial DNA (mtDNA). Mitochondria

are commonly used in studies of phylogenetics because they have their own circular genome which is separate from the nuclear chromatin (Boore 1999). The matrix of the mitochondria contains the genetic material, including DNA, RNA and ribosomes. This study focuses on determination of the utility of the COI gene as a DNA "barcode" for both indigenous and non-indigenous ascidian species in Newfoundland. DNA "barcoding" aims to provide an accurate method for species-level identifications, offers alternative approaches to studying biodiversity, and is useful for ecological surveys and environmental assessments (Bhadury et al. 2006; Hajibabaei et al. 2007). DNA "barcoding" uses a short segment of the DNA, approximately 15-20 bases long, to identify species. There have been numerous studies showing that COI can be used to differentiate a wide range of taxa at the species level (Armstrong and Ball 2005). In most cases COI has been proven effective for species identification in many animals including fish, copepods, insects and birds (Hebert et al. 2004; Ward et al. 2005; Hajibabaei et al. 2006; Pfenninger et al. 2007).

Genetic markers are important for early detection because ascidian eggs and larvae are difficult to identify based on morphology. Once the barcode regions are identified, genetic markers can be developed for use in environmental monitoring of these species in Newfoundland as well as other coastal areas.

1.7 OBJECTIVES

This study investigates the biofouling community of marine megainvertebrates on wharf pilings in four Newfoundland harbours (Argentia, Botwood, Corner Brook and

Port-aux-Basques). The objectives of this research are (a) to determine if known, non-indigenous species of ascidians are present in the harbours (Chapter 2), (b) to describe the species composition, abundance and biodiversity of the megabenthic fouling invertebrate community (Chapter 2), and (c) to determine the nucleotide sequence of the COI gene and its variability in indigenous and non-indigenous ascidian species (Chapter 3). The community data presented in Chapter 2 is valuable as baseline information for the detection of future invasions. This tool can be used for rapid and early detection of potential future introductions of these non-indigenous species in and around Newfoundland.

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1.9 CO-AUTHORSHIP STATEMENT

The research described in this thesis was carried out by Ashley G. Callahan, with guidance from Drs. Don Deibel, Cynthia MacKenzie and Matthew Rise, and from Jennifer Hall. Ashley G. Callahan was responsible for the data collection and analysis.

Authorship for Chapter 2: Callahan, A.G., D. Deibel and C.H. McKenzie.

Authorship for Chapter 3: Callahan, A.G., D. Deibel, J. Hall, M. Rise, C.H. McKenzie.

CHAPTER 2: HARBOUR SURVEYS AND MEGABENTHIC COMMUNITY ANALYSIS FOR INDIGENOUS AND NON-INDIGENOUS INVERTEBRATES

2.1 INTRODUCTION

Invasive species are one of the leading environmental issues in the world (Stachowicz et al. 2002). It is important for managers to know the indigenous species present in their waters so that if non-indigenous species are detected, regulations and management programs can be implemented to help control their spread (Campbell et al. 2007). Ascidians are marine invertebrates that live in all oceans. They are filter-feeding, hermaphroditic organisms that have the ability to reproduce quickly when conditions are favourable. Their larva are short-lived, thus limiting their capability to spread long distances and colonize new areas (Svane and Young, 1989; Lambert and Lambert 1998; Locke et al. 2007). Because of their limited dispersal potential, anthropogenic vectors are important in the spread of these organisms. The main transport vectors for ascidians include shipping traffic at multiple spatial scales where ascidians may be transported attached to the ship hulls as juveniles and adults, or in the ballast water as larvae (Svane and Young 1989; Lambert and Lambert 2003). Another vector that is important in the spread of ascidians is through the transfer of shellfish among aquaculture sites (Carver et al. 2003).

Invasive, non-indigenous ascidians have become a significant biofouling problem

for the aquaculture industry in Nova Scotia and Prince Edward Island since the mid-1990's (Howes et al. 2007; Locke et al. 2007) The non-indigenous and invasive ascidians causing problems in Atlantic Canada include *Styela clava*, *Ciona intestinalis*, *Botryllus schlosseri* and *Botrylloides violaceus*.

Artificial structures are prime locations for the establishment of non-indigenous ascidians. Invasive ascidians are a problem because they compete with and can overgrow indigenous benthic communities, fouling boats, floating docks and attaching to mussel and oyster aquaculture infrastructure (Lambert and Lambert 1998; Carver et al. 2003; LeBlanc et al. 2003). They can cause reduced productivity of aquaculture industries by competing for space and restricting water flow and thus food availability for bivalves (Carver et al. 2003). Invasive ascidians may also affect aquaculture operations by increasing production costs due to increasing handling time, requiring additional equipment and increasing the physical demands on crew (Carver et al. 2003; LeBlanc et al. 2003, Howes et al. 2007, Locke et al. 2007). In addition, there are increased husbandry costs due to extra cleaning and processing time.

Given the high level of vessel traffic between Newfoundland and the Maritime Provinces, it is likely that one or more of these species will invade Newfoundland harbours. It is important to track these non-indigenous ascidians because the blue mussel aquaculture industry in Newfoundland has expanded rapidly in the past five yeas, providing rural employment.

This study is the first assessment of non-indigenous invertebrates in Newfoundland harbours. The goals of this project were to use quadrat samples, visual surveys and photographic records to assess the abundance and biodiversity of

megainvertebrates on wharf pilings and to determine if non-indigenous ascidians are present in four high-risk ports. All four ports are visited regularly by a variety of ships sailing from locations in the southern Gulf of St. Lawrence as well as from the northeastern United States and Europe.

2.2 MATERIALS AND METHODS

2.2.1 Study Sites

Newfoundland is an island located on the east coast of Canada. A survey of four Newfoundland harbours (Argentia, Botwood, Corner Brook and Port-aux-Basques) was conducted in September-November 2006 (Trip 1) and November-December 2006 (Trip 2) (Fig. 2.1). These harbours are considered to be at high risk for the invasion of non-indigenous ascidians (Therriault and Herborg 2008a; Therriault and Herborg 2008b). Argentia, Corner Brook and Port-aux-Basques were chosen because there are high levels of shipping traffic from Nova Scotia and Prince Edward Island. This shipping traffic includes ferries that run from Argentia and Port-aux-Basques to North Sydney, Nova Scotia. Botwood was chosen as a study harbour because of its proximity to several Newfoundland mussel farms. Several ascidian species are known to foul mussel aquaculture lines in other Atlantic Provinces so it is important to know if non-indigenous ascidians are present in this area. The 'Corner Brook' sampling harbour included a wharf located in Lark Harbour on the Humber Arm, 48 km from the main Corner Brook wharf, and another at Frenchman's Cove, also on Humber Arm, 25 km from Corner Brook wharf (Fig. 2.1). Sampling was not conducted in Frenchman's Cove during Trip

2. These alternative sites were required due to our inability to obtain access to commercial wharves within the inner Corner Brook harbour.

2.2.2 Sampling

'Scrape' samples were taken by SCUBA divers using a 0.25 cm^2 quadrat frame (Stachowicz et al. 2002; Grey 2009) from 3 sites on each of 2-3 wharves in each harbour (Fig. 2.2). Sites were chosen by determining the perimeters of each wharf counter clockwise using a tape measure, then multiplying the perimeter by a random number from a table ranging from 0.01-1. This number gave the distance of each site from the point of origin. This procedure was followed with the following caveats: 1. In the case of any obstruction that precluded quadrat placement, a new random number was chosen. 2. If the resulting sites were within three meters of each other, a new random number was chosen. 3. If the water depth was less than three meters, a new random number was chosen. All sample sites were recorded using GPS and the location on the wharf was permanently labeled so it could be revisited during Trip 2.

The quadrat frame was positioned at each site on a wharf piling at a nominal depth of 2 m below the lowest low tide. All macroalgae and megafauna were removed from the piling within the frame by a SCUBA diver using a putty knife and placed into a bag with 1 cm mesh. For Trip 2, the scrape samples were taken adjacent to the previous scrapings at the same sites. After the samples were transported ashore, the contents of the bag were transferred to 10 L plastic buckets filled with sea-water. The mesh bags were examined to ensure that any attached invertebrates or algae were retained with the

sample. At the end of the day, each sample was poured into a plastic tray and photographed to provide a permanent record. These photographs were not analyzed quantitatively. When present, up to three individuals of each species of tunicate were removed from each sample and placed in 95% ethanol to be used in genetic analyses (see Chapter 3). The remaining sea-water in the bucket was poured though a mesh sieve (450 μm) to collect organisms. The material on the screen was washed into a bucket with filtered sea-water and the contents fixed in a 10% sea-water formaldehyde-solution. Environmental data (temperature, salinity, turbidity, dissolved oxygen, light) was collected for each harbour, although this data will not be presented here.

2.2.3 Image Analysis

Underwater photographs were taken of each quadrat before the area was scraped. The underwater camera was held by hand as near to the center of each grid as possible. In the laboratory, a 9 x 9 virtual mesh grid (i.e. 81 points) was overlaid over each quadrat frame image with using Image-J (NIH). Images that were out of focus or non-orthogonal were not analyzed. Of the 30 scrape samples from Trip 1, 21 had corresponding photographs of sufficient quality to be analyzed. Percent cover was estimated using the point-transect method (Pecha et al. 2004), by identifying the taxon of marcoalgae or megafauna underlying each grid point and then summing the number of grid points per taxon for each photograph. As we used 81 grid points, each point represented a cover of 1.2 %. If the photograph included a non-natural component within part of the grid frame (such as a diver's glove), the number of points overlying the obstruction was subtracted

from 81 and the percentage represented by each grid point adjusted accordingly. Flora (algae, seaweeds, etc), fauna (invertebrates of all sizes including bryozoans and hydroids), 'substrate' (wharf pilings or artificial structures in which material was attached) and 'unknown' (targets that could not be identified or were out of focus) were included in the percent coverage estimates and were grouped together as 'other'.

In addition to the photographs of quadrats described above, a vertical series of images was taken at each sampling site from the surface of the water to the seafloor in order to provide a qualitative record of the vertical distribution of visible fauna on the wharf pilings. This imaging was done along a taut line at 50 cm intervals. Each photograph was then examined on a computer monitor and a species list of all identifiable macroalgae and megafauna was compiled. The taxonomic composition of each site was compared for each of the three sampling methods which included quadrat scrape samples, underwater photographs of the quadrat frames before the samples were removed, and the vertical series along the taught line. This combination of sampling techniques gives a more complete record of the megafauna community of each site, increasing the likelihood of detecting rare non-indigenous species and decreasing ambiguities of interpretation of taxa in the photographs.

2.2.4 Laboratory and Statistical Analyses

In the laboratory, the organisms from each scrape sample were sorted into major taxonomic groups (typically family and order) and transferred to 95% ethanol (Mastrototaro et al. 2008). The organisms were identified using several taxonomic keys

(Gosner 1925; Plough 1978; Pockington 1989; Pollock 1997). Abundance of megafauna taxa at each site, wharf, and harbour was determined for organisms collected on a 1 mm sieve. In addition, epifauna (i.e. *Balanus* spp. and *Stronglyocentrotus droebachiensis*) greater than or equal to 1 cm were scraped off *Mytilus* spp., seaweed, etc. and then processed through the 1 mm sieve. The taxa were organized into the following groups for Trip 1. '*Mytilus* spp.' which includes *Mytilus edulis* and *Mytilus trossulus,* 'Sea stars' which includes *Asterias* spp. and *Ophiopholis aculeate,* 'Ascidians' which includes *Molgula* spp. and *Ascidia* sp. *and* 'Polychaetes' which includes *Polynoidae* and *Hesionidae.* The groups were the same for Trip 2 except that 'Ascidians' includes *Molgula* spp., *Ascidia* sp. and *Halocynthia pyriformis,* and 'Polychaetes' which includes *Lepidonotus squamatus*, *Polynoidae* and *Hesionidae, Hesionidae nereimyra, Terebellidae,* and *Polycirrus* spp.

The number of individuals in each taxon in each sample was multiplied by 16 and are reported here as individuals m^{-2}. Mean abundance (± standard deviation) of taxa was calculated for each wharf and harbour. Only sites where taxa were present were used to calculate mean values (i.e. 0 values were excluded from calculation of the means). As a double check medians were calculated with the zero values included. Community parameters such as the number of taxa, number of individuals per taxon, Shannon-Wiener's diversity index (*H'*) and Evenness (J) were calculated for each harbour.

2.2.5 Multivariate Community Analysis

Differences in taxonomic composition and abundance of the megafaunal

community among wharves within harbours and among the four harbours were explored using non-metric multidimensional scaling (nMDS) (PRIMER-E v6 software) based on the Bray-Curtis similarity index (Clarke and Gorley 2006). nMDS was useful in this study since it takes into account both the changes in abundance and species composition and can resolve subtle differences in community structure (Ugland et al. 2008).

The number of individuals in each taxon in each sample from Trip 1 and Trip 2 were pooled and then were multiplied by 8 and are reported as individuals m^{-2}. The 'wharf' factor included wharves A1, A2, B1-B3, C1-C2, D1-D3, and the 'harbour' factor included A, B, C, D, where 'A' is Argentia, 'B' is Botwood, 'C' is Corner Brook, and 'D' is Port-aux-Basques. The abundance data was log (x+1) transformed to reduce the effect of highly abundant taxa and increase sensitivity to rare taxa. The Bray-Curtis index and resultant similarity matrix was used to determine similarities between samples (Wilber et al. 2007). The resemblance matrix was computed using zero-adjusted Bray-Curtis similarity, which adds a dummy variable of 1 to the abundance matrix to adjust for cells with values of 0 (Clarke and Gorley 2006; Clarke et al. 2006). A dummy variable is analogous to adding a 'dummy species' to the matrix and forcing two samples with no content to be 100% similar (Clarke and Gorley 2006).

The statistical significance of faunal clusters was determined with the CLUSTER group average function in PRIMER-E. nMDS was performed using the MDS function based on the similarity matrix; the number of algorithms was increased from the default of 25 to 50. Cluster analysis output and nMDS plots were compared by overlaying contour lines (cluster similarity: 20 and 70%) on the corresponding nMDS graph. The stress value associated with the nMDS plot reflect how well the distance among samples

in the plot reflect actual distances (Clark and Gorley 2006; Quijon and Snelgrove 2006). ANOSIM (analysis of similarity) tests were performed using a two-way nested ANOVA using the factors wharf and harbour. This tests the null hypothesis that there is no difference between wharfs and harbours. Using PRIMER-E ANOISIM, R-values > 0.5 indicate significant differences between groups (Clarke and Gorley 2006; Wilber et al. 2007). The SIMPER function in PRIMER-E was used to determine the average similarity and dissimilarity of the individual species contributing to the separation between the harbours based on a one-way analysis with harbour as the factor.

2.3 RESULTS

2.3.1 General Community Composition

Using all three sampling methods resulted in the collection of 22 different taxa, 19 of which were identified to the species level (Table 2.1). Thirteen taxa were detected using the quadrat scrape method, four were documented in the quadrat scrape images, and ten others in the images from the vertical series. The taxa fell into the following groups, the Cnidaria (1 family), Annelida (4 families), Arthropoda (3 families), Mollusca (7 families), Echinodermata (3 families) and Tunicata (3 families). The molluscs and annelids contained the most species identified (i.e. 7 and 6 species, respectively, Table 2.1).

2.3.2 Scrape Sample Analysis

The samples from Trip 1 (September-October 2006) showed that *Mytilus* spp. were numerically dominant in all four harbours, with mean abundances ranging from 1120 - 6523 indivduals m^{-2} (Table 2.2). The second and third most abundant taxa were *Stronglyocentrotus droebachiensis* and *Balanus* spp., with abundances of 392 and 272 individuals m^{-2}, respectively. *S. droebachiensis* and ascidians were found only in Corner Brook and Port-aux-Basques, while *Balanus* sp. and sea stars were only found in Argentia. *Metridium senile* and polychaetes were found in all harbours except Botwood. It is interesting to note that *Mytilus* spp. were the only species found in the scrape samples in Botwood and the only species that were observed in all four harbours.

The samples from Trip 2 (October-December 2006) showed similar results to Trip 1, evidence that the species presence and abundance patterns were consistent (Table 2.3). Mussels were again present in all four harbours, with mean abundances of 176 – 4429 individuals m^{-2}. The second and third most abundant taxa were ascidians in Argentia and polychaetes in Corner Brook, with abundances of 208 individuals m^{-2} and 200 individuals m^{-2}, respectively. Ascidians and polychaetes were observed in Argentia in addition to the two harbours where they were sampled during Trip 1. Ascidians were not detected in samples collected at Argentia during Trip 1. The ascidian species found during both trips were *Molgula* sp. and *Ascidia* sp. and are indigenous to Newfoundland (Gretchen Lambert pers. comm.). Sea stars were found in two additional harbours not reported during Trip 1, Corner Brook and Port-aux-Basques.

2.3.3 Scrape Samples versus Quadrat Images

Comparing the results from the scrape samples to the photographic images of the quadrats revealed several differences between the two sampling methods. Figs. 2.3 and 2.4 show the occurrence of taxa in each harbour from the scrape samples from Trips 1 and 2, while Fig. 2.5 shows the distribution of taxa from the quadrat images from Trip 1. There were 13 megafaunal invertebrate taxa identified in the scrape samples, compared to only 4 taxa from the photographic analysis of the quadrats. The difference in numbers of taxa identified could be a result of the fact that many of the taxa identified in the samples were rare with low mean abundances, and therefore were likely underestimated by our 81-point, line-transect image analysis routine. Also note that macroalgae covered by far the greatest amount of area in all of the quadrat photographs (data not shown). Macroalgal identification in the foregoing scrape samples was not an objective of my project.

2.3.4 Scrape Samples versus Vertical Rope Series Images

The vertical series of photographs allowed us to make a qualitative record of the vertical zonation of taxa at the four harbours. *Mytilus* spp. were the only taxa that were present in both the scrape samples and in the vertical photographic series in all four harbours (Table 2.4). *Balanus* spp. was only detected in the vertical series in Corner Brook and Port-aux-Basques, but was detected in both the scrape samples and vertical series in Argentia. This could indicate that *Balanus* sp. were located in Corner Brook and

Port-aux-Basques below the nominal sampling depth of the scrape samples and photographs. There were only two species (*Coryphella* sp. and *Thyasira* sp.) identified in the quadrat scrapes that were not identified in the vertical series. This discrepancy could be a result of the fact that the quadrat scrapes were at an ideal depth for sampling these species. Alternatively, these two taxa may have been too small or too rare to be identified via the vertical photographic series. There were no taxa identified only in the vertical photographic series in comparison to the scrape samples or quadrat photographs.

2.3.5 Diversity and Evenness

The Shannon-Wiener diversity index and evenness values were calculated based on grouping taxa into phylogenetic orders from Trips 1 and 2 (Table 2.5). Prior to analysis, sea star taxa were grouped together and included two orders (Forcipulatida and Ophiurida), which included *Asterias* spp. and *Ophiopholis* sp. The diversity and evenness values were very similar from Trips 1 and 2. The highest diversity and evenness values were in Argentia (H'= 1.15, J'= 0.72; H'= 1.47, J'=0.91) for Trip 1 and 2, respectively. Corner Brook and Port-aux-Basques had diversity indexes less than 0.50 and evenness values less than or equal to 3.71. Diversity and evenness could not be calculated for Botwood because there was only one species present.

2.3.6 Multi-variate Community Analyses

A 2-D nMDS plot provided an acceptable representation of the community

composition and abundance, with a stress level of 0.11 (Fig. 2.6). The primary result was that Argentia was different from the other three harbours based on the relative distances between points. Based on the CLUSTER analysis, wharves in Argentia grouped together at only a 20% similarity level, indicating a relatively high level of variability among wharves. Two Corner Brook wharves were included in the Argentia cluster, with one of them grouping with an Argentia wharf at 70% similarity. The three harbours of Botwood, Corner Brook and Port-aux-Basques were clustered together at a level of 20% similarity (Fig 2.6). Within this cluster, Botwood showed the greatest similarity among wharves, with a majority included in a single group at 70% similarity. Corner Brook and Port-aux-Basques were more similar to each other than they were to Botwood, grouping at 70% similarity. ANOSIM analysis indicated no significant difference between wharves within harbours ($R=0.13$, $p > 0.05$), while there was a significant difference between harbours ($R=0.52$, $p < 0.01$).

This difference between harbours was further investigated using SIMPER, which indicated that the dissimilarity ranged from 48 to 88 % (Table 2.6). Argentia was > 82% dissimilar from the other 3 harbours, supporting the clusters identified in Fig. 2.6. There were several species contributing to the differences between the harbours, including *Mytilus* spp. (accounting for 22 – 41 % of the total difference), sea stars (12 – 17 %), *Strongylocentrotus droebachiensis* (14 %), *Balanus* sp. (11 %), polychaetes spp. (8 %) and *Metridium senile* (6 %) (Table 2.7). *Mytilus* spp. contributed the most to dissimilarity among harbours, with one exception. *S. droebachiensis* contributed the most to dissimilarity between Botwood and Port-aux-Basques, with a value of 14 %. Polychaetes only contributed to the dissimilarity between Corner Brook and Port-aux-Basques.

2.4.7 Invasive and Non-Indigenous Ascidians

No non-indigenous ascidians were detected during this survey. However, invasive *Botryllus schlosseri* was observed on the hull of a small boat in Argentia during a parallel Rapid Assessment Survey by SCUBA divers on December 7, 2006 (McKenzie and Deibel, unpubl.). This exemplifies the utility of quadrat studies for quantitative estimates of relatively common species and Rapid Assessment Surveys for the qualitative detection of potentially invasive species, which may be too rare to be detected in a quadrat survey. The indigenous ascidians *Mogula* sp. and *Ascidia* sp. were detected in the quadrat samples. In addition to the samples, indigenous *Halocynthia pyriformis* were detected in the vertical photographic series. Another indigenous ascidian, *Boltenia echinata,* was observed by SCUBA divers during the parallel Rapid Assessment Survey.

2.4 DISCUSSION

To my knowledge this is the first assessment of the megainvertebrate fouling community of wharf pilings in Newfoundland. Thus, specific literature comparsions are difficult. The abundance and types of organisms found during this study are similar to fouling organisms reported from elsewhere in the western Atlantic Ocean (Greene and Grizzle 2007). Our study revealed that *Mytilus spp.*, which included the blue mussel (*Mytilus edulis*) and the bay mussel (*Mytilus trossulus*), are the most abundant and

common species among the harbours. Mussels are known to be competitive dominants and abundant members of fouling communities (Mathieson et al. 1991; Greene and Grizzle 2007). In Greene and Grizzle's (2007) study of successional development of fouling communities in the Gulf of Maine, *Mytilus edulis* was the most abundant in density and biomass. Botwood had the highest abundance of mussels compared to any of the other harbours in this study. This could be due to the fact there are many mussel aquaculture sites in this region that are constantly seeding their farms with new mussel spat. There is the possibility that some of this spat is being released into the water, therefore seeding the surrounding areas including the wharves in Botwood harbour (B. Lowen, pers. comm.).

Other species that were abundant members of the fouling community in Newfoundland harbours, as indicated through the various assessment methods used, were the green sea urchin, *Stronglyocentrotus droebachiensis*, *Metridium senile, Balanus* spp., sea stars and polychaetes. The seastar *Asterias vulgaris*, and the anemone *Metridium senile,* are also found in high numbers/and or biomass in the Gulf of Maine (Greene and Grizzle 2007). Several of the rare taxa found in the scrape samples in this study included *Hiatella* spp., *Thyasira* spp. and amphipods. Whereas in the Gulf of Maine, amphipods and *Hiatella artica* had the highest biomass during certain periods of the year, i.e. September-October and June-July (Greene and Grizzle 2007).

The major source of variability in community species composition and abundance was differences among harbours, rather than among wharves within harbours. This is likely due to the large geographic area over which the harbours are spread (Fig. 2.1), as well as to the very different oceanographic environment in the region of each harbour, i.e.

the Gulf of St. Lawrence for Corner Brook and Port-aux-Basques, the Gulf Stream and shelf waters for Argentia, and the Labrador Current for Botwood. Perhaps not surprisingly then, these latter two harbours are most distinct. Argentia is the most taxonomically diverse harbour and is significantly different from the other three harbours in terms of community composition and abundance (nMDS analysis, Fig. 2.6). Botwood was distinct in terms of being almost entirely dominated by mussels. These clear differences among harbours should provide a valuable biotic community baseline of information against which future changes in these harbours can be assessed.

The only non-indigenous ascidian species found was *Botryllus schlosseri,* which was discovered in Argentia harbour during a parallel Rapid Assessment Survey (McKenzie and Deibel, unpubl.). It is interesting to note that Argentia is the only harbour in which a non-indigenous species was found during this study. In a recent assessment, Argentia was ranked as one of Newfoundland's ports at highest risk for invasion (Baines 2007). According to a shipping report from Transport Canada in 2000, ca. 47% of the shipping traffic entering Newfoundland was from the eastern seaboard of the USA, while ca. 42% came from Europe and the Mediterranean Sea (Balaban 2000). As well there is a regular ferry that travels from Argentia to Nova Scotia from June to September.

Argentia harbour is located in Placentia Bay, which is on the south coast of Newfoundland (Fig. 2.1). Within Placentia Bay, there is another port (Come by Chance/Whiffen Head) which is located approximately 60-80 km from Argentia. This port receives large oil tankers that release ballast water in the area and many of these come from a region affected by *B. schlosseri* invasions (Baines 2007). The distribution of *B. schlosseri* can also be affected by secondary transport. There is much fishing and

recreational boating activity in the bay, and *B. schosseri* has the potential to attach to the hulls of small boats. This is problematic because these vessels have the potential to transport *B. schosseri* to smaller bays and inlets where larger ships do not travel. Also, there is no governmental data base of traffic information on small vessels. Importantly, the *B. schlosseri* found in Argentia was attached to the hull of a small boat that had been docking there for over a year. This tunicate was previously noted on the south coast of Newfoundland in 1975 (Hooper 1975), but has not been reported from other regions of the island in subsequent years until this project began in 2006. The federal government took the discovery of *B. schlosseri* in Placentia Bay seriously, prohibiting the export of mussels from the bay for processing in 2006. This highlights the importance of non-indigenous species issues in Newfoundland and the need for more scientific information to better inform management decision making.

I used three different methods to determine the megabenthic invertebrate fouling community of wharf pilings. Some of the methods (line photographs) were more effective in the detection of rare species while others (quadrat scrape samples) were more effective in quantitative estimation. In the future, more Newfoundland harbours and aquaculture sites should be surveyed by SCUBA divers in order to detect non-indigenous ascidians using Rapid Assessment Survey techniques. This was a pilot study. If it were repeated, it would be useful to increase both the size and number of quadrats if the goal is to detect and to quantify rare non-indigenous species.

2.5 CONCLUSIONS

In conclusion, there are many species that contribute to the megabenthic invertebrate fouling community in Newfoundland harbours. Although the potentially invasive ascidians are not yet common members of these communities, it is important to continuously monitor these areas and other high risk harbours. There is a high possibility that the four non-indigenous ascidians in Atlantic Canada, *Styela clava, Ciona intestinalis, Botryllus schlosseri* and *Botrylloides violaceus,* will become common members of benthic communities in Newfoundland harbours in the years to come. This 2006 survey confirms the presence of *B. schlosseri* in Newfoundland for the first time since 1975.

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Table 2.1. List of benthic megafauna invertebrate taxa observed in the study using three sampling methods (quadrat scrapes, and quadrat and vertical rope photographic series, see Methods).

Phylum	Class	Sub-Class	Order	Family	Species
Cnidaria	Anthozoa	Zoantharia	Actiniaria	Metridiidae	*Metridium senile*
Annelida	Polychaeta	Palpata	Aciculata	Polynoidae spp.	
				Polynoidae	*Lepidonotus squamatus*
				Hesionidae	Hesionidae spp.
					Hesionidae nereimyra
			Canalipalpata	Terebellidae	*Polycirrus* spp.
				Serpulidae	*Spirorbis* spp.
Arthropoda	Malacostraca	Eumalacostraca	Decapoda	Pandalidae spp.	
			Amphipoda	Amphipod spp.	
		Cirripedia	Thoracica	Balanidae	*Balanus* spp.
Mollusca	Gastropoda		Neotaenioglossa	Littorinidae	*Littorina* spp.
					Littorina littorea
			Patellogastropoda	Acmaeidae	*Acmaea* spp.
		Opisthobranchia	Nudibranchia	Coryphellidae	*Coryphella* spp.
	Bivalvia	Pteriomorphia	Mytiloida	Mytilidae	*Mytilus* spp.
		Heterodonta	Mytoida	Hiatelloidea	*Hiatella* spp.
			Veneroida	Thyasiridae	*Thyasira* spp.
Echinodermata	Stelleroidea		Ophiurida	Ophiatidae	*Ophiopholis aculeata*
			Forcipulatida	Asteriidae	*Asterias* spp.
	Echinoidea		Echinoida	Strongylocentrotidae	*Strongylocentrotus droebachiensis*
Chordata	Ascidiacea		Stolidobranchia	Pyuridae	*Halocynthia pyriformis*
				Molgulidae	*Mogula* spp.
				Ascidiidae	*Ascidia* spp.

Table 2.2. Mean abundance (individuals m^{-2}) of megafauna invertebrate taxa* from four Newfoundland harbours from quadrat scrapes from September 2006 and October 2006 (Trip 1). Only sites where taxa were present were used to calculate means. Values in square brackets are the number of sites a taxa was present ('n') out of the total number of sites per harbour. Errors are reported as (standard deviation), except when $n \leq 3$ and is reported as half the range. (- - -) = none observed in sample.

	Number of quadrats per harbour	*Mytilus spp.*[a]	*Stronglyocentrotus droebachiensis*	Sea stars[b]	*Metridium senile*	Ascidians[c]	*Balanus* spp.	Polychaetes[d]
Argentia	6	1120 [1]	(- - -)	64 (56) [3]	37 (24) [3]	(- - -)	272 (144) [2]	48 [1]
Botwood	9	6523 (5743) [9]	(- - -)	(- - -)	(- - -)	(- - -)	(- - -)	(- - -)
Corner Brook	6	2468 (1343) [4]	392 (72) [2]	(- - -)	72 (56) [2]	48 [1]	(- - -)	96 (0) [2]
Port-aux-Basques	9	2354 (2934) [8]	57 (55) [7]	(- - -)	112 (104) [3]	88 (72) [2]	(- - -)	112 (16) [2]

[a] *Mytilus* spp. includes *Mytilus edulis* and *Mytilus trossulus*
[b] Sea stars includes *Asterias spp.* and *Ophiopholis aculeata*
[c] Ascidians includes *Molgula* spp. and *Ascidia sp.*
[d] Polychaetes includes *Polynoidae* and *Hesionidae*

*Rare taxa which are not represented above include:
Corner Brook: *Coryphella* sp. (16 m^{-2}), [1/6]

Table 2.3. Mean abundance (individuals m^{-2}) of megafauna invertebrate taxa* from four Newfoundland harbours from quadrat scrapes from October 2006 and December 2006 (Trip 2). Only sites where taxa were present were used to calculate mean. Values in square brackets are the number of sites a taxa was present out of the total number of sites per harbour. Errors are reported as standard deviation, except when $n \leq 3$ and is reported as half the range. (- - -) = none observed in sample.

	Number of quadrats per harbour	***Mytilus* spp.[a]**	***Stronglyocentrotus droebachiensis***	**Sea stars[b]**	***Metridium senile***	**Ascidians[c]**	***Balanus* spp.**	**Polychaetes[d]**
Argentia	6	176 [1]	(- - -)	100 (68) [4]	32 (16) [2]	208 [1]	88 (8) [2]	(- - -)
Botwood	9	4429 (3154) [9]	(- - -)	(- - -)	(- - -)	(- - -)	(- - -)	(- - -)
Corner Brook	3	3120 (48) [2]	32 [1]	16 [1]	(- - -)	64 [1]	(- - -)	200 (104) [2]
Port-aux-Basques	9	1367 (960) [9]	76 (48) [8]	48 [1]	32 [1]	48 (40) [3]	(- - -)	64 (32) [2]

[a] *Mytilus spp.* includes *Mytilus edulis* and *Mytilus trossulus.*
[b] Sea stars includes *Asterias* spp. and *Ophiopholis aculeata.*
[c] Ascidians includes *Molgula* sp., *Ascidia* sp. and *Halocynthia pryiformis.*
[d] Polychaetes includes *Lepidonotus squamatus*, *Polynoidae* and *Hesionidae, Hesionidae nereimyra, Terebellidae,* and *Polycirrus* spp.

*Rare taxa which are not represented above include:
Botwood: *Hiatella* (16, [1/9]), Snail (16, [1/9]), Amphipod (16, [1/9])
Corner Brook: *Thyasira* (16, [1/3])
Port-aux-Basques: (*Hiatella* sp.and *Thyasira sp.* 16,[1/9]), Shrimp (16, [1/9]); *Coryphella* sp. (16, [1/9])

Table 2.4. Comparison of megafauna invertebrate taxa identified from quadrat scrapes and vertical rope series. '+' indicates presence and '-' indicates absence.

	Argentia		Corner Brook		Port-aux-Basques	
Species	Scrape	Rope	Scrape	Rope	Scrape	Rope
Mytilus spp.	+	+	+	+	+	+
Stronglyocentrotus droebachiensis	-	-	+	-	+	+
Metridium senile	+	-	+	+	+	+
Sea Stars	+	+	-	-	+	+
Balanus spp.	+	+	-	+	-	+
Coryphellidae spp.	-	-	+	-	+	-
Ascidian	-	+	+	-	+	+
Amphipod	-	-	-	-	-	+
Polychaetes	+	+	+	-	+	+
Hiatella spp.	-	-	-	-	+	-
Thyasira spp.	-	-	+	-	-	-
Littorina spp.	-	-	-	+	-	-

[a] *Mytilus spp.* includes *Mytilus edulis* and *Mytilus trossulus.*
[b] Sea stars includes *Asterias* spp. and *Ophiopholis aculeata.*
[c] Ascidians includes *Molgula* sp. or *Ascidia* sp.
[d] Polychaetes includes *Lepidonotus squamatus*, *Polynoidae* and *Hesionidae, Hesionidae nereimyra, Terebellidae,* or *Polycirrus* spp.

Table 2.5. Shannon-Wiener diversity index (H') and evenness (J) of quadrat scrape samples from four Newfoundland Harbours from Trip 1 and 2. The H and J could not be calculated for Botwood Trip 1 because there was only one species present and calculations cannot be computed on one species. Sea star orders combined (see text).

	Trip 1		**Trip 2**	
Harbour	**H'**	**J'**	**H'**	**J'**
Argentia	1.15	0.72	1.47	0.91
Botwood	0	0	0.01	0.01
Corner Brook	0.44	0.25	0.60	0.37
Port-Aux-Basques	0.30	0.31	0.38	0.17

Table 2.6. The dissimilarity percentages of taxa between harbours, calculated using the SIMPER function in PRIMER-E®.

	Argentia	Botwood	Corner Brook	Port-aux-Basques
Argentia		88	83	82
Botwood			58	48
Corner Brook				60
Port-aux-Basques				

Table 2.7. The individual taxa contributing to the dissimilarity between harbours shown in Table 2.6, including the average dissimilarity percentages, the % contribution of each taxon to the total dissimilarity, and the cumulative %.

Comparsion	Taxon	Average Dissimilarity (%)	% Contribution	% Cumulative
Argentia vs. Botwood	*Mytilus spp.*	40	46	46
	Sea Stars	17	20	66
Argentia vs. Corner Brook	*Mytilus spp.*	22	26	26
	Sea Stars	18	21	47
	Balanus spp.	11	14	61
Argentia vs. Port-aux-Basques	*Mytilus spp.*	23	28	28
	Strongylocentrotus droebachiensis	14	17	45
	Sea Stars	12	14	60
Botwood vs. Corner Brook	*Mytilus spp.*	30	52	53
Botwood vs. Port-aux-Basques	*Strongylocentrotus droebachiensis*	14	30	30
	Mytilus spp.	8	17	46
	Metridium senile	6	13	59
Corner Brook vs. Port-aux-Basques	*Mytilus spp.*	16	27	27
	Strongylocentrotus droebachiensis	13	22	49
	Polychaetes	8	13	62

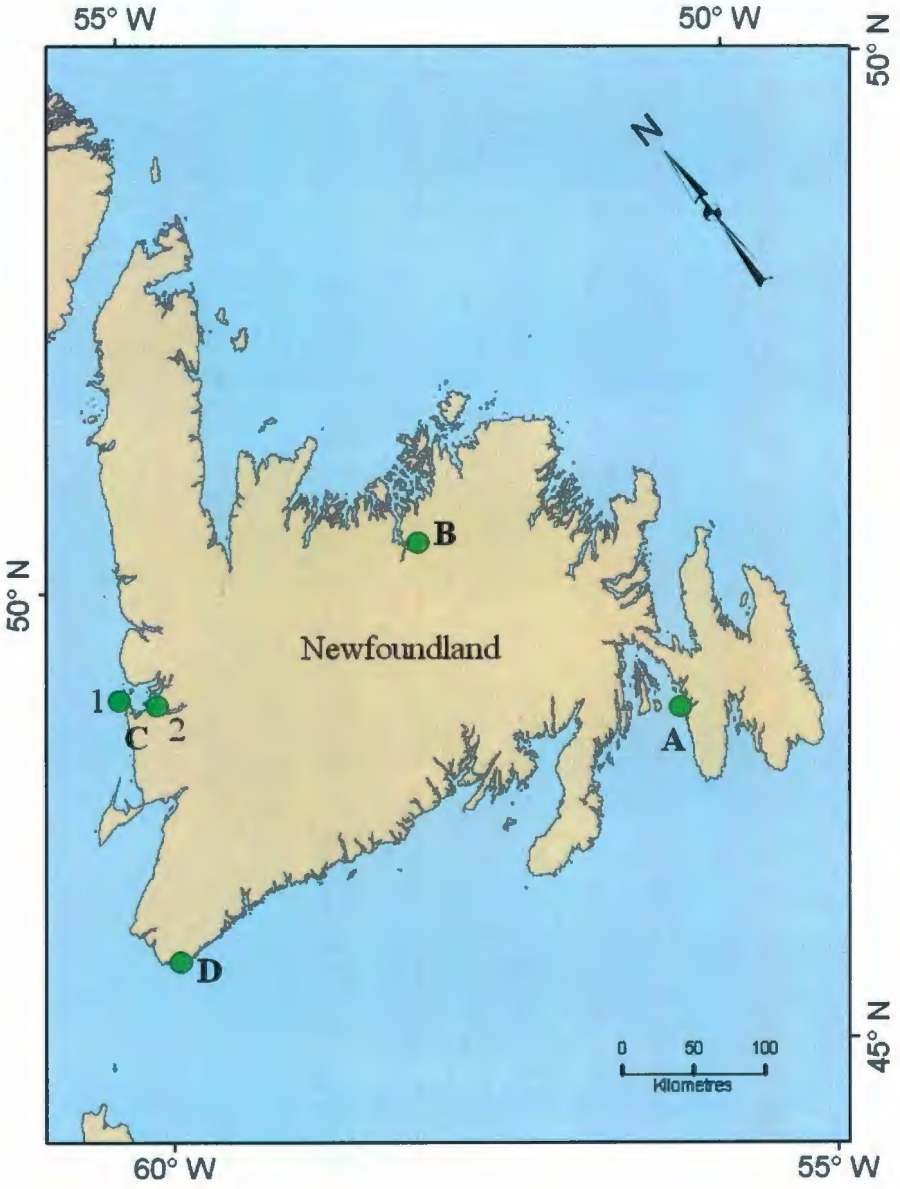

Figure 2.1. Map of insular Newfoundland, Canada, showing the sampling harbours. A= Argentia; B= Botwood; C= Corner Brook (1= Lark Harbour and 2= Frenchman's Cove); D= Port-aux-Basques (map courtesy of R. Brushett).

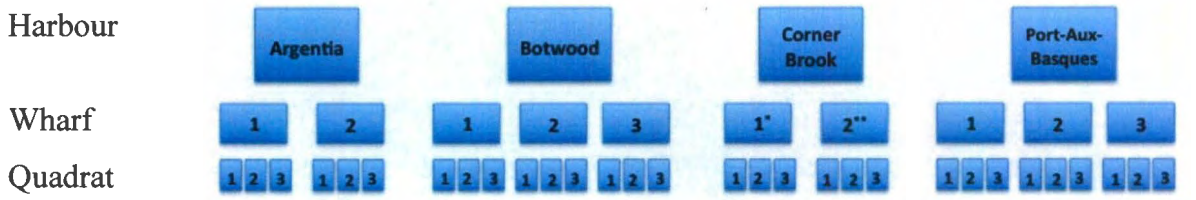

Figure 2.2. Diagrammatic representation of the experimental design

During Trip 2 Corner Brook (Frenchman's Cove) was not sampled.

* Lark Harbour

** Frenchman's Cove

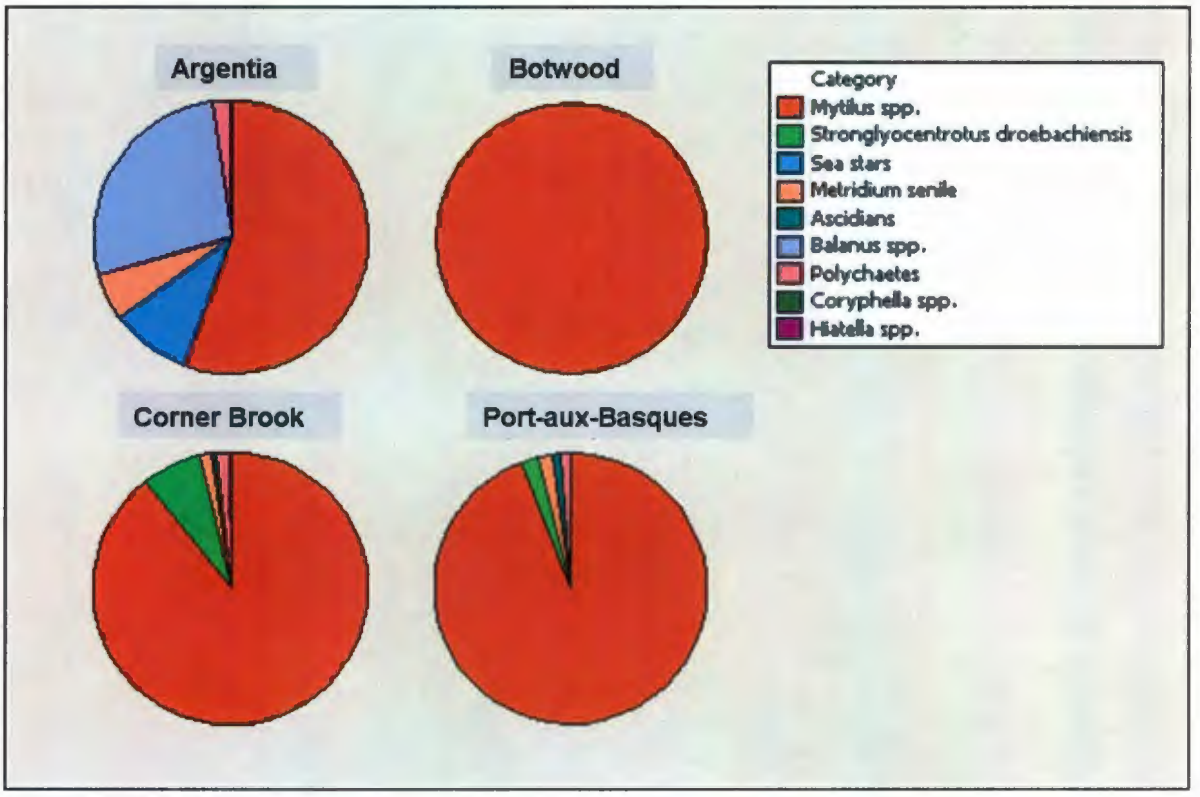

Figure 2.3. Occurrence of each of taxon from quadrat scrapes from Trip 1 (September 2006-October 2006).

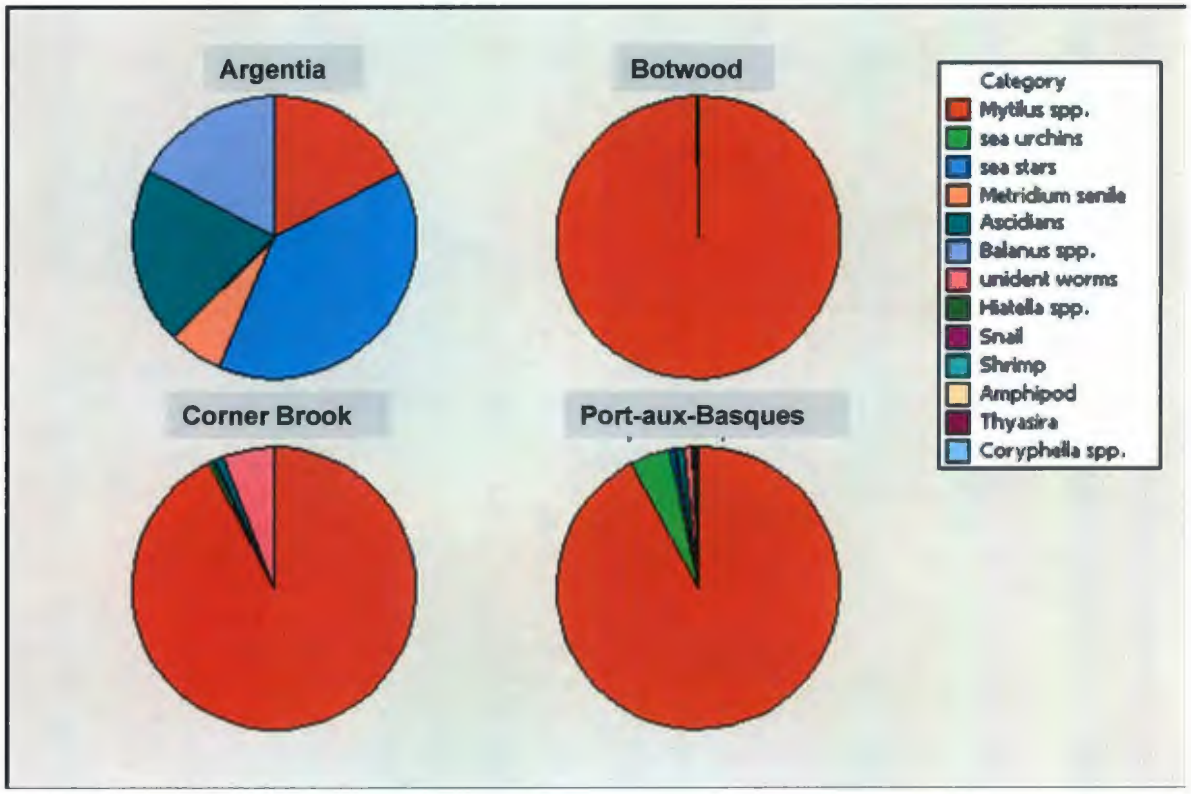

Figure 2.4. Occurrence of taxon from quadrat scrapes from Trip 2 (October 2006-December 2006).

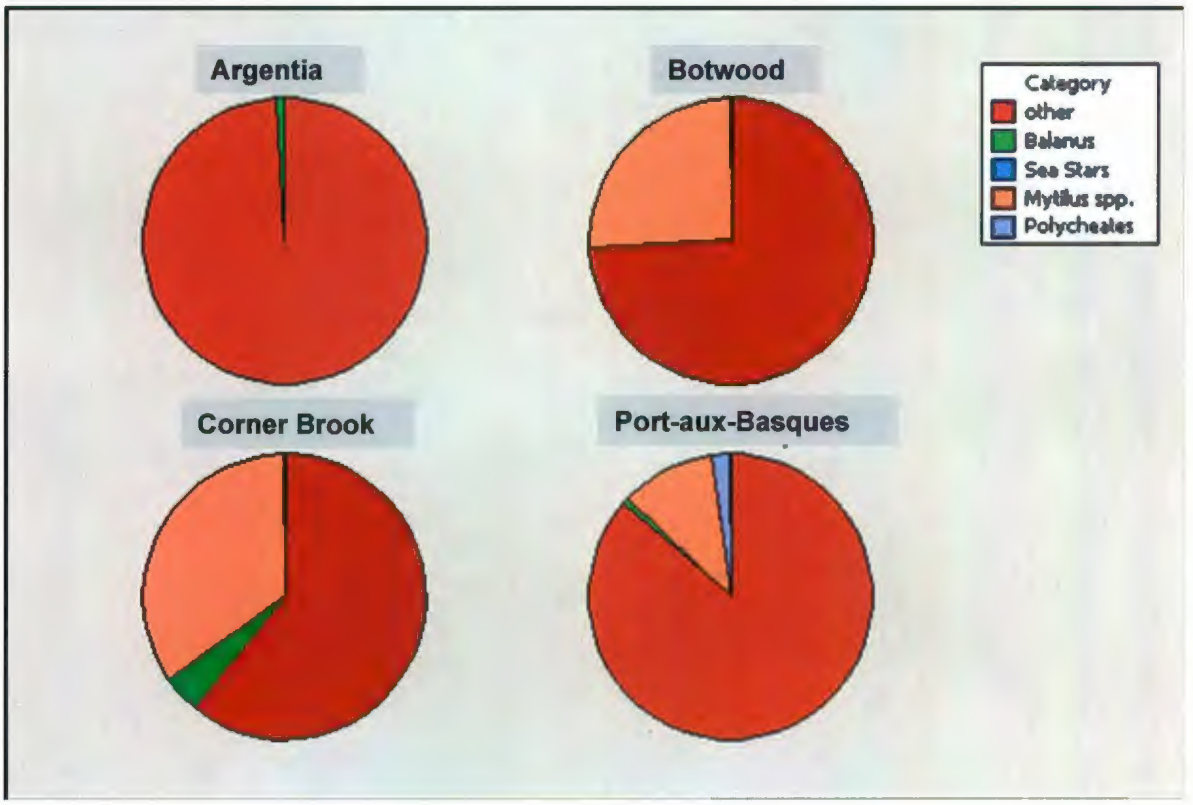

Figure 2.5. Occurrence of taxon from quadrat images scrapes from Trip 1 (September 2006-October 2006).

The 'other' category includes: flora (algae, seaweeds, etc), fauna (invertebrates of all sizes including bryzoans and hydroids), 'substrate' (wharf pilings or artificial structures in which material was attached) and 'unknown' (targets that could not be identified or out of focus sections of the pictures.

The number of pictures analyzed varied per harbour. The total number of pictures per harbour is as follows: Argentia = 4; Botwood = 8; Corner Brook = 4; Port-aux-Basques = 5.

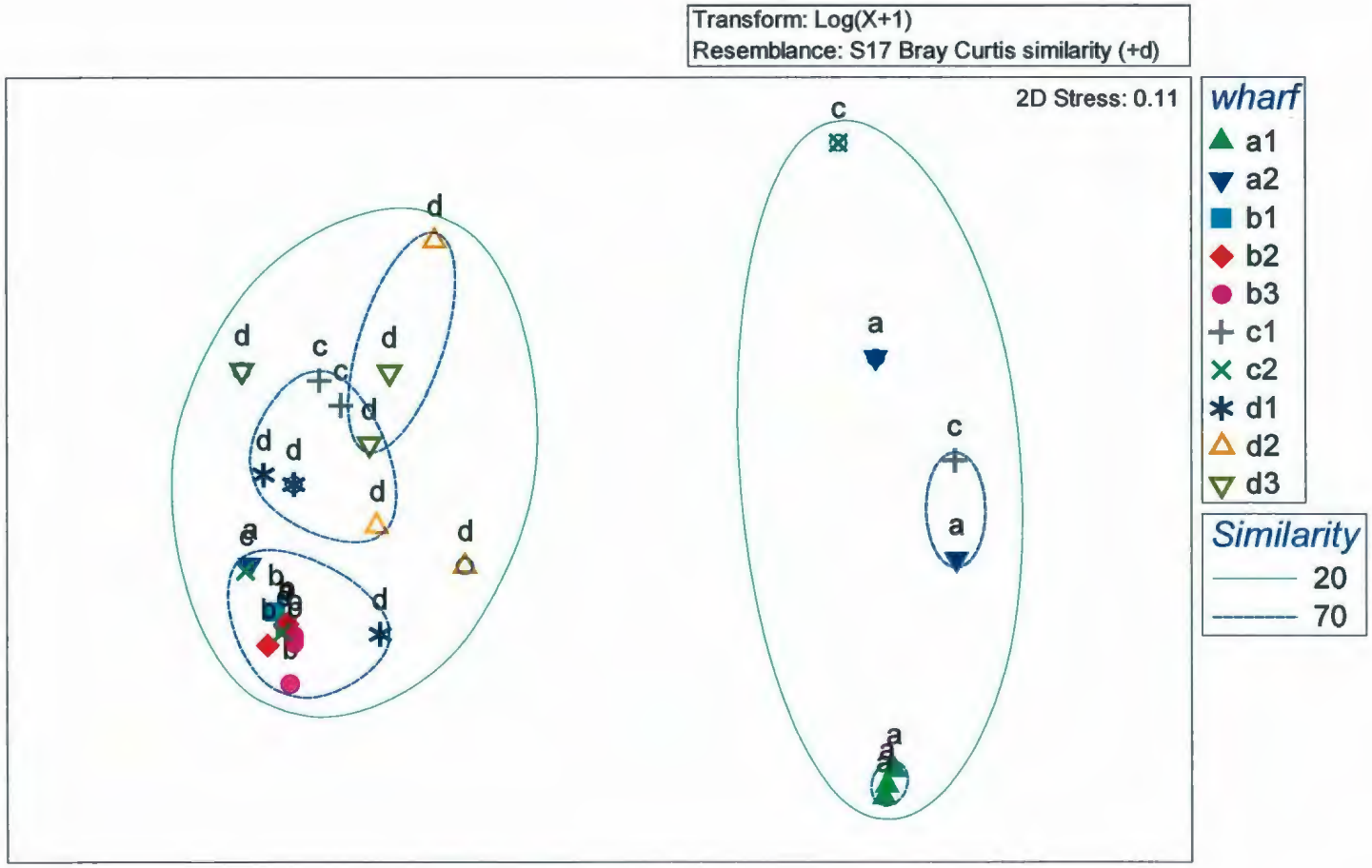

Figure 2.6. Two dimensional nMDS plot of the community composition and abundance of megainvertebrates in four Newfoundland harbours (Argentia 'a', Botwood 'b', Corner Brook 'c', and Port-aux-Basques 'd'). Similarity to each other was determined using the nMDS mode in PRIMER. The dotted (blue) and circular lines (green) encircling the treatments 'wharves' have been plotted using the CLUSTER function based on the Bray-Curtis similarity index with similarity of values of 20 and 70%.

CHAPTER 3: DETERMINATION OF CYTOCHROME *C* OXIDASE GENE SEQUENCES OF INDIGENOUS AND NON-INDIGENOUS ASCIDIAN TUNICATES OF NEWFOUNDLAND.

3.1 INTRODUCTION

Several non-indigenous ascidian species (*Styela clava, Ciona intestinalis, Botryllus schlosseri* and *Botrylloides violaceus*) have become a major biofouling problem in the Canadian Maritime provinces in the past ten years (LeBlanc et al. 2003; Locke et al. 2007). Non-indigenous ascidians (NIA) foul both artificial and natural substrates. Shipping vectors are likely important in the introduction of non-indigenous species such as ascidians, which may be transported to new areas through ballast water, sea chests or attached to hulls (Svane and Young 1989; Carlton and Geller 1993; Carver et al. 2003; Lambert and Lambert 2003 and Locke et al. 2007). However, secondary spread of these species via small boats is also a problem and it is difficult to prevent spreading to smaller harbours and inlets by local vessel traffic (Wasson et al. 2001).

There are many shipping linkages between the Canadian Maritimes and Newfoundland. Of the four known invasive ascidian species in the Maritime Provinces, two of them, *Botryllus schlosseri* and *Botrylloides violaceus,* have been detected in Newfoundland in the past two years. *B. schlosseri* was detected in December 2006 in Argentia, on the bottom of a small boat (McKenzie and Deibel, unpubl.). Subsequently, it has been detected during additional surveys in many harbours in Placentia Bay and surrounding areas (Fig. 3.1). This is the first report of *B. schlosseri* in Newfoundland

since 1975 (Hooper 1975). *B. violaceus* was detected in September 2007 on the south coast in Belleoram harbour (McKenzie and Deibel, unpubl.). This is the first report of *B. violaceus* in Newfoundland.

In addition to the non-indigenous ascidians in Newfoundland, there are several common indigenous species that inhabit natural and artificial substrates. These species include *Boltenia echinata, Halocynthia pyriformis, Molgula* sp., and *Aplidium* sp. (Van Name 1945; Plough 1978).

In this study, two potentially invasive species (*Botryllus schlosseri* and *Botrylloides violaceus*) were analyzed genetically. *B. schlosseri* and *B. violaceus* are potentially invasive, colonial ascidians, with mixed development and short larval periods, typically < 2 d (Stachowicz et al. 2002). *B. schlosseri* is a now a cosmopolitan species that originated from the Mediterranean Sea, while *B. violaceus* it thought to have originated from the Northwest Pacific Ocean (Berrill 1950; Carver et al. 2006). For comparison, the two indigenous ascidians *Boltenia echinata* and *Halocynthia pyriformis* were also analyzed. *B. echinata* and *H. pyriformis* are solitary ascidians, both having a northern boreal distribution (Plough 1978).

Generally, benthic marine species with pelagic larvae are considered to have little population genetic structure because of the wide dispersal potential of the larvae (Palumbi 1994; Dias et al. 2006;Yuan et al. 2009). However, ascidian tunicates may differ from this pattern, because they have very short-lived larvae and can have considerable genetic structure even on spatial scales < 10 m (Yund 1995; Ayre et al. 1997; López-Legentil et al. 2006; Demarchi et al. 2008). This limited dispersal could make the identification of species-specific genetic markers difficult, because within-

species genetic variability may become nearly as large as among species variability. For example, the COI gene is known to be highly polymorphic in several species of ascidians (Tarjuelo et al. 2003; Tarjuelo et al. 2004; López-Legentil et al. 2006; Silva and Smith 2008). Ascidians are difficult to identify with morphological methods, especially when they are in the egg, larval and juvenile stages of development (Darling and Blum 2007).

The objectives of this study were a) to develop a method for determination of the nucleotide sequence of the COI gene of mtDNA of indigenous and non-indigenous ascidian tunicates collected in Newfoundland, b) to determine whether these sequences can be used to distinguish among the species of Newfoundland ascidians, i.e. whether nucleotide sequence variability among species is greater than within species. This information is required for future use of genetic markers for molecular identification of eggs and larvae of invasive ascidians in Newfoundland, in order to evaluate whether the gene sequences are species-specific (Ward et al. 2008) And c) to compare Newfoundland haplotypes within each species to haplotypes from other samples I have collected in North America and to GenBank sequences from North America, the eastern Atlantic Ocean and the Mediterranean Sea to confirm my taxonomic identification of the Newfoundland specimens and to construct a geneology of the non-indigenous species to develop hypotheses on possible source populations. This information is required if vectors of invasion are to be identified and managed in the future.

3.2 METHODS

3.2.1 Sample Collection

In 2006 and 2007, adult specimens of four ascidian species of ascidians were collected from sites within Newfoundland, Prince Edward Island and Massachusetts by SCUBA divers or by hand from wharf pilings. The two non-indigenous ascidians to Newfoundland that were sequenced were *Botryllus schlosseri* and *Botrylloides violaceus*, while the indigenous ascidians were *Halocynthia pyriformis* and *Boltenia echinata*. *B. schlosseri* was collected from four harbours in Newfoundland (Fig. 3.1, North Harbour [B], Hermitage [A], Argentia [D], Arnold's Cove [C]), one harbour in Woods Hole, MA (J) and one harbour in the Murray River, Prince Edward Island (I). One colony of *B. schlosseri* was sequenced from each of the four locations in Newfoundland and two individuals were sequenced from both Prince Edward Island and MA (Table 3.1). *B. violaceus* was collected in one Newfoundland harbour (Fig. 3.1, Belleoram [H]), one harbour in Woods Hole, MA and one harbour in Murray River, Prince Edward Island. Three individuals of *B. violaceus* were sequenced from Newfoundland, one from PEI and three from MA (Table 3.1). Four individuals of *H. pyriformis* were sequenced from Port-aux-Basques (Fig. 3.1, [E]) and one was sequenced from Logy Bay (Fig. 3.1, [F], Table 3.1). One individual of *B. echinata* was sequenced from each location Port-aux-Basques, Logy Bay and Bauline, Newfoundland. Ascidian species were fixed in 95% ethanol and stored at room temperature.

3.2.2 DNA Extraction, COI Amplification and Sequencing

Ascidians were identified visually before genetic analysis was performed (Plough

1978; Pollock 1997). Several zooids (~5-10) from a cluster were extracted for the colonial ascidians and considered one sample. Tissue was extracted from the pharyngeal sac for the solitary ascidians. Total DNA was extracted from ascidians using the DNeasy Blood & Tissue Kit (Qiagen Inc.) following the manufacturer's protocol. Before DNA was extracted, ascidian tissue samples were soaked in a series of ethanol/phosphate buffered saline (PBS) washes as follows for five minutes each: 1. 75% ethanol/25% PBS, 2. 50% ethanol/50% PBS, 3. 25% ethanol/75% PBS and 4. 100% PBS. Three primer sets were used for the amplification of a fragment of the mtDNA COI gene. The primer sets included the universal primers, HCO2198r, 5'- TAA ACT TCA GGG TGA CCA AAA AAT CA- 3', LCO1490f, 5'- GGT CAA CAA ATC ATA AAG ATA TTG G- 3' designed by Folmer et al. (1994), ASC_COI_F, 5'- TCG ACW AAT CAT AAA GAT ATT AG- 3', ASC_COI_R ,5'- GTA AAA TAA GCT CGA GAA TC-3' (Vogler, per. comm.), and a novel primer set was developed for *Botrylloides violaceus*, Violet Forward, 5'-TTA GGT TTT GGT CTA GGT TTA TTG-3', Violet Reverse, 5'-TAA ATG TTG ATA AAG TAC AGG GTC-3'.

Polymerase chain reaction (PCR) amplifications were performed in 50µl total reaction volumes containing 1µl (10 µM) of each primer, 1µl (10mM) dNTPs, 5µl 10X buffer containing 15 mM $MgCl_2$, 1µl (1,000 units/ml) Dynazyme , and 1µl-10µl (~ 100 ng) template DNA. A single incubation at 94°C for 2 min was followed by 40 cycles of (94°C for 30 sec, 37°C for 30 sec, and 72°C for 1 min) and a final extension at 72°C for 7 min, on an ABI GeneAmp PCR system 9700 thermocycler. PCR products were then separated by 1.5% agarose gel electrophoresis, and bands were excised and purified using the QIAquick Gel Extraction Kit (Qiagen Inc.). The PCR amplified COI fragments were

then subcloned into pGEM T-Easy Vector Systems (Promega) and then transformed using either Subcloning Efficiency DH5Alpha Competent Cells (Invitrogen) or JM109 High Efficiency Competent Cells (Promega) following the manufacturer's instructions. Recombinant clones were screened for inserts of correct size and positives were cultured and purified using the QIAprep Miniprep Kit (Qiagen). Clones from each individual were then sequenced in both directions in an ABI 3730xl automated sequencer using M13 forward and M13 reverse primers.

3.2.3 Sequence Variability and Genetic Distance

Clones for each individual were compiled and aligned using Vector NTI Advance 10. Alignments were confirmed by inspection and a consensus sequence was composed for each individual ascidian COI fragment. Compilation and alignments were performed using AlignX in Vector NTI Advance 10, which uses the CLUSTAL W algorithm (Thompson et al. 1994).

The genetic diversity measures, i.e. haplotype and nucleotide diversity and the number of polymorphic sites, were calculated for the four ascidian species sequenced in this study using ARLEQUIN version 3.0 (Schneider et al. 2000). Due to the low sample size, individuals from each species were grouped into larger populations before diversity analyses. Individuals of *Botryllus schlosseri* and *Botrylloides violaceus* were combined into two groups, Newfoundland and the Northwest Atlantic (Woods Hole, Massachusetts + Prince Edward Island), whereas individuals of *Halocynthia pyriformis* and *Boltenia*

echinata were combined into a Newfoundland group.

Sequence difference was calculated to quantify genetic variability within and among the four ascidians. Pairwise genetic distance (100 – the sequence identity score) was calculated pair-wise for all haplotypes using AlignX. Haplotype codes are shown in Table 3.1.

Sequences of *Botryllus schlosseri* from this study were compared with those in GenBank to determine the geneological relationships of Newfoundland samples of *Botryllus schlosseri* to those from the Northwestern Atlantic, the Northeastern Atlantic and the Mediterranean Sea. All sequences longer than 524 bp were trimmed using AlignX to a final partial sequence length of 524 bp to ensure that all sequences were evualated over the same length. Whenever possible, haplotype codes follow those of López-Legentil et al. (2006). Haplotype codes and GenBank numbers are shown in Table 3.1. All sequences were aligned and imported in MSF format into MEGA version 4.1 (Kumar et al. 2001) and the relationshipd among haplotypes was determined using the maximum likelihood method with Tajima-Nei distance (Tajima and Nei, 1984). Bootstrap analysis was performed with 1000 replicates. To confirm the haplotype relationships we also constructed the two parameter (K2P) distance model of Kimura (1980). Both methods gave similar results.

3.3 RESULTS

3.3.1 COI Sequences of Newfoundland Species

I obtained partial sequences of the mitochondrial cytochrome *c* oxidase I (COI) gene from four species of Newfoundland ascidians from 8 different harbours (i.e.,populations) (Table 3.1). The universal primers developed by Folmer et al. (1994) for the amplification of the COI gene were tried on all four species, *Halocynthia pyriformis, Boltenia echinata, Botrylloides violaceus* and *Botryllus schlosseri*. These primers amplified well and amplicons yielded identifiable COI sequences for *H. pyriformis* and *B. schlosseri*, but yielded poor or incorrect sequences for *B. echinata* and *B. violaceus*. Therefore, another primer set developed by Vogler (pers. comm.) was applied to these two species. These Vogler primers were successful in the amplification of COI in *B. echinata* but failed for *B. violaceus.* Therefore, primers were designed for *B. violaceus* based on the limited sequences using from the Folmer primers. The *B. schlosseri* partial COI sequence that I amplified was 658 bp long, whereas the sequences of *B. schlosseri* obtained from GenBank ranged from 524 bp to 674 bp. The *B. violaceus* partial COI sequence I amplified was 590 bp, whereas those of *H. pyriformis* and *B. echinata* were 658 bp. Those sequences which have been submitted to GenBank are indicated in Table 3.1. My sequences are the first in GenBank for *H. pyriformis* and *B. echinata*. The lengths reported are the sequences between the primer regions.

The four species sequenced in this study were compared with those in GenBank using BLAST analysis and p-distance to confirm taxonomic identification. In the cases of *Halocynthia pyriformis* and *Boltenia echinata*, there were no other sequences available in GenBank at the time of data analysis. However, they did match closely with other ascidians in the Pyuridae family. *H. pyriformis* matched closely (85% similarity) with the well-studied ascidian, *Halocynthia roretzi*. Pérez-Portela (2009) reports sequence data

for *H. pyriformis* from Havre St Pierre, Quebec, which is the same (i.e. 100 % similarity) as the PH1 haplotype reported in this study. *Boltenia echinata* was most closely related to P*yura praeputialis* at 78.1 percent similarity. *Botryllus violaceus* and *Botryllus schlosseri* matched closely with other ascidians in the Styelidae family. Upon comparing my *B. violaceus* sequences to other ascidians in GenBank, the most closely-related species was *B. schlosseri* (82 % similarity). This was interesting because Pérez-Portela et al. (2009) report sequence data on *B. violaceus* in GenBank which differs considerably from my *B. violaceus* sequence (only 80 % similarity). This indicates that the specimens may have been misidentified by Pérez-Portela et al. (2009) before sequencing or mis-catalogued in GenBank. Further support for the accuracy of my sequences are that I found only one haplotype and no genetic diversity within samples of *B. violaceus* from the NWA, which agrees with the findings of Bock et al. (2009). *B. schlosseri* matched other *B. schlosseri* sequences, a finding which is discussed in detail below.

3.3.2 COI Sequence Variability of Newfoundland Species

All measures of genetic diversity were much higher among individual *Botryllus schlosseri* from Newfoundland harbours than among individuals of the other three species examined in this study (Table 3.2). In fact, genetic diversity of *B. schlosseri* was higher in Newfoundland populations than in samples from the Northwestern Atlantic (NWA) (i.e. NWA, Woods Hole, Mass. and Murray R., Prince Edward Island), with 3 haplotypes and 29 polymorphic sites in Newfoundland compared with only 1 haplotype among the NWA populations. In comparison to *B. schlosseri*, genetic diversity of the

non-indigenous *Botrylloides violaceus* was much lower, with only one haplotype shared among the Newfoundland and NWA populations (Table 3.2).

Genetic diversities of the indigenous species *Halocynthia pyriformis* and *Boltenia echinata* were intermediate between that of *Botryllus schlosseri* from Newfoundland and *Botrylloides violaceus* (Table 3.2). Newfoundland populations of each of the two indigenous species had two haplotypes, ≤ 2 polymorphic sites and a mean haplotype diversity of 0.40 – 0.67. The mean haplotype and nucleotide diversity of *B. schlosseri* was significantly greater than the mean values for *H. pyriformis* ($p < 0.05$) but not significantly different from those of *B. echinata* ($p > 0.05$).

The proportion of nucleotide sites at which sequences were different (i.e. p-distance) was lower among haplotypes within each species than among species (Table 3.3). The range of values within species was < 1 % for all species except *Botryllus schlosseri*, which ranged from <1 to 15.6 %, including the haplotypes from GenBank listed in Table 3.1 (i.e., including European haplotypes in the comparison). The among-species ranges in p-distance were all > 17.7 %, with 5 of the 6 ranges having minimum values > 20 % (Table 3.3).

3.3.3 Gene Genealogy of Botryllus schlosseri

Sequences of *Botryllus schlosseri* from this study were compared with those in GenBank to determine the phylogenetic relationships of Newfoundland populations to those from the Northwestern Atlantic, the Northeastern Atlantic and the Mediterranean Sea (see Fig. 3.3; Table 3.1 for a list of samples in each group). Of the 3 Newfoundland

haplotypes, 2 are relatively closely-related to populations elsewhere in the NWA, whereas the North Harbour population had no closely related haplotype elsewhere in the NWA region. The HJ haplotype, shared by Grana (NEA) and Blanes (MED), Spain, appears to represent an intermediate haplotype leading to Arnold's Cove, Argentia and Hermitage, Newfoundland populations. This finding indicates a mixed NEA and MED origin of these 3 Newfoundland genotypes. The Hermitage population is somewhat differentiated from the HJ lineage however, closely resembling the HO haplotype from Rochelle, France (NEA). The North Harbour, Newfoundland, haplotype is quite divergent from the other Newfoundland haplotypes, showing a great deal of affinity to several haplotypes from the Mediterranean Sea, and only 1, from Grana, Spain in the NEA. North Harbour is only 18 km from Arnold's Cove. The bottom half of Fig. 3.3. contains haplotypes that are relatively more divergent from those in the NWA and Newfoundland, indicating very little trans-Atlantic gene flow of these haplotypes.

3.4 DISCUSSION

Mitochrondrial (mtDNA) COI sequences of four ascidian species, *Botrylloides violaceus, Botryllus schlosseri, Halocynthia pyriformis* and *Boltenia echinata,* were obtained from Newfoundland and the Northwestern Atlantic. The four species differed markedly in the susceptibility of the COI gene to PCR amplification using the typical, universal Folmer primers. Whereas *H. pyriformis* and *B. schlosseri* worked well with Folmer primers, *B. violaceus* and *B. echinata* did not. The Folmer primers are commonly-used PCR primers for universal metazoan invertebrates and have been used successfully

to amplify over 80 taxa including ascidians and bivalves (Folmer et al., 1994; Castilla et al., 2002; Stach and Tubverville 2002; Kappner and Bieler 2006; Lopez-Legentil 2006; Turon and Lopez-Legentil 2004; Curole 2004). Pérez-Portela et al. (2009) reported failure of amplification in *Stolonica socialis* (Stolidobranchia ascidian) using the Folmer primers, and therefore created a new pair of specific primers to amplify the COI gene in this species. However, they reported no problem with the amplification of *B. violaceus* using the Folmer primers. Bock et al. (2009) also report sequence data for *B. violaceus* obtained using the Folmer primers. In this study, the yielding of poor or incorrect sequences could be a result of this universal primer amplifying DNA from a non-target organism. This was likely since many of the ascidians collected were attached to other organisms (i.e. *Mytilus* spp.). In the case of *B. violaceus,* the new primer set developed worked successfully the majority of the time in the amplification of this species. The new primer was developed by aligning the partial sequences of *B. violaceus* and other closely related species to identify regions in the sequences that were different. The Vogler primer set is a modification of the Folmer primers that are intended to be more specific for the amplification of COI in ascidians. This set was successful in the amplification of several ascidians species from the Mediterranean (Vogler, per. comm.) and our *Boltenia echinata*.

Sequence information provided confirmation of our taxonomic identifications. In the cases of *Halocynthia pyriformis* and *Boltenia echinata*, although there were no other sequences available in GenBank, they matched most closely with other ascidian species in their Pyuridae family. My sequence information from *Botrylloides violaceus* suggests a possible misidentification of this species in GenBank (Pérez-Portela et al. 2009). This

type of error can occur frequently in GenBank records (Pérez-Portela et al. 2009). Excepting this possible misidentification, my sequence will be the first for *B. violaceus* in GenBank and my *B. schlosseri* sequences will add to the information in GenBank.

The genetic diversity measures varied among the four species in this study. Diversity was much higher in *Botryllus schlosseri* compared to the other three ascidian species. There were three haplotypes found for *B. schlosseri* from four samples sequenced from Newfoundland, whereas there were two haplotypes for the other species. In combining the haplotypes in this study and the sequences in GenBank, 24 haplotypes were found in 184 samples. Of course, relatively low sample number can mean I have underestimated intra-specific spatial genetic differentiation in Newfoundland and the NWA (Ward et al., 2008). However, my estimates of the number of haplotypes falls within the range of that published for *B. schlosseri* by López-Legentil et al. (2006) of 2-4 population^{-1} based on sample sizes of 11-25. They found three haplotypes from only four samples at Woods Hole, Massachusetts, U.S.A., as I did for *B. schlosseri* in Newfoundland (Table 3.2). The number of polymorphic sites found for *B. schlosseri* within Newfoundland (29) was much higher than the polymorphic sites in the other three ascidians (0-2) from this study. However, they do fall within the range of the polymorphic sites for *B. schlosseri* (12-89) reported by Lopez-Legentil (2006). High numbers of polymorphic sites have also been reported for other ascidians, *Ciona intestinalis, Ciona savignyi*, *Cytodytes* sp., *Clavelina lepadiformis* and *Pseudodistoma* sp. (Turon et al. 2003; Tarjuelo et al. 2001; Lopez-Legentil and Turon 2005; Silva and Smith 2008). There are high levels of polymorphism reported for the invasive ascidians *C. intestinalis and C. savignyi* from California (Dehal et al., 2002; Vinson et al. 2005). Silva

and Smith (2008) reported recently that ten different ascidian species show substantially different levels of genetic polymorphism and exceptions to the assumptions that invasive species start with a low level of genetic polymorphism that increases over time.

The high genetic diversity of the COI gene in *Botryllus schlosseri* must be taken into account during species-specific molecular marker development. It is vital for there to be less nucleotide variation between individuals within a species than between individuals of different, even closely-related species (Darling and Blum 2007). The high percent difference range within *B. schlosseri* was close to the range of differentiation between *B. schlosseri* and *B. violaceus*. Intra-specific variation is rarely greater than 2%. This high degree of variability in *B. schlosseri* could be a result of geographical isolates (Hebert et al., 2003). The lack of overlap between inter and intra-specific distributions of genetic variation is crucial to successful species-level assignments (Meyer and Paulay 2005; Darling and Blum 2007).

In contrast to *Botryllus schlosseri,* the other non-indigenous ascidian *Botrylloides violaceus* showed no genetic diversity (i.e. there was only one haplotype, BV1) within Newfoundland and the NWA. Given that *B. violaceus* has been present in the NWA for at least the past ten years (Locke et al. 2007) and has only been discovered in Newfoundland recently, it can be inferred that *B. violaceus* most likely arrived in Newfoundland from the east coast of the U.S.A. These results are comparable to those of Bock et al. (2009), who also found very low haplotype and nucleotide diversity in *B. violaceus*. Within a total of 192 samples from North America and Europe there were only five haplotypes and the individuals from the NWA all shared the same haplotype (Bock et al. 2009).

When *Botryllus schlosseri* haplotypes from this study were compared to those obtained from GenBank, our results indicated that there are relationships between Newfoundland and the NWA samples to sequences from Europe, indicating that there is gene flow in this ascidian between the two continents. The three haplotypes from Newfoundland were all collected within a relatively small geographical area, in and around Placentia Bay. Within this small area it was surprising to find relatively high genetic variability within a low sample size. Since ascidians cannot disperse long distances on their own and have short-lived planktonic larva, they must have been introduced to Newfoundland via shipping traffic (Svane and Young, 1989). It is likely that this shipping traffic originated from populations within the NWA, the NEA and the MED. It can be inferred that *B. schlosseri* in Newfoundland came from different source populations and probably through multiple introductions. This interpretation fits with general biological invasion trends in that most biological invasions result from multiple introductions. Human mediated dispersal also tends to promote higher levels of within-population genetic diversity (Wilson et al., 2008). Given that the HO haplotype from Hermitage was shared with individuals from Rochelle Harbour, France and Maine, USA, these could be potential source populations. In contrast, because the HA haplotype from North Harbour shared the same haplotype as individuals from the Mediterranean Sea, this is likely the source population. In addition, the other haplotype (BS1) collected in Placentia Bay from Argentia and Arnold's Cove shared a haplotype with individuals from the NWA. The HJ haplotype seems to be an intermediate haplotype between the two continents, separating the NWA clade and the NEA and MED clades. The results of this study are comparable to those of Bock et al. (2009), who propose that east coast

populations of *B. schlosseri* have a Mediterranean Sea origin.

In addition to shipping as a vector for the introduction of *Botryllus schlosseri* to the NWA, human-mediated transport of cultivated bivalves has been hypothesized to be a major source of range expansion within and between Mediterranean Sea and eastern Atlantic populations of the nassarid snail *Cyclope neritea* (Couceiro et al. 2008). Since I have observed invasive ascidians growing on mussel shells in Newfoundland, the transportation of bivalve aquaculture products may also account for the high level of relatedness among *B. schlosseri* populations in the Mediterranean Sea and eastern Atlantic (López-Legentil et al. 2006). It will be important in future research to sequence more individuals from several Newfoundland populations to expand knowledge of haplotype diversity and to help pinpoint source populations of *B. schlosseri* and *Botrylloides violaceus* in Newfoundland. This question has important management implications, because regulation of invasion vectors is often the most effective way to control the spread of invasive marine invertebrates (Bax et al. 2003).

3.5 REFERENCES

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Table 3.1. Summary of collection information for the ascidian species studied. Included are the geographic location name, map code for Figs. 3.1 and 3.2, latitude and longitude (GPS), sample size (n), haplotype code and GenBank accession numbers. Species shown in bold face were determined in this study.

Species	Location	Map Code	Latitude	Longitude	n	Haplotype code	GenBank Accession no.
			Newfoundland (NL)				
Botrylloides violaceus	**Belleoram**	H	**47.5272**	**-55.4092**	**3**	**BV4**	**GU065355[a], GU065356[a]**
Botryllus schlosseri	**Hemitage**	A	**47.5563**	**-55.9259**	**1**	**HO**	**GU065354[a], DQ340216[b]**
	North Harbour	B	**47.8590**	**-54.1000**	**1**	**HA**	**GU065353[a], DQ340205[b]**
	Arnold's Cove	C	**47.8747**	**-54.1682**	**1**	**BS1**	**GU065350[a]**
	Argentia	D	**47.2920**	**-53.9904**	**1**	**BS1**	**GU065349[a]**
Halocynthia pyriformis	**Port-aux-Basques**	E	**47.5751**	**-59.1402**	**4**	**PH1**	**EU178858[a]**
	Logy Bay	F	**47.6253**	**-52.6646**	**1**	**PH2**	**EU178861[a]**
Boltenia echinata	**Port-aux-Basques**	E	**47.5751**	**-59.1402**	**1**	**BE1**	**GU065360[a]**
	Logy Bay	F	**47.6253**	**-52.6646**	**1**	**BE2**	**GU065361[a]**
	Bauline	G	**47.7232**	**-52.8348**	**1**	**BE2**	**GU065362[a]**
			North West Atlantic (NWA)				
Botrylloides violaceus	**Murray River, PEI**	I	**46.0170**	**-62.6155**	**1**	**BV1**	**GU065357[a]**
	Woods Hole, MA	J	**41.5170**	**-70.6683**	**3**	**BV1**	**GU065358[a], GU065359[a]**
Botryllus schlosseri	**Murray River, PEI**	I	**46.0170**	**-62.6155**	**2**	**BS1**	**GU065351[a]**
	Woods Hole, MA	J	**41.5170**	**-70.6683**	**2**	**BS1**	**GU065352[a]**
	Woods Hole, MA	J	41.2103	-70.6683	4	HQ, HR, HS	DQ340222[b], DQ340223[b], DQ340224[b]
	Maine, USA	K	44.4133	-68.7300	1	HO	DQ367525
			North East Atlantic (NEA)				
Botryllus schlosseri	Rochelle Harbour, France	L	46.1442	1.17027	18	HO	DQ340216[b]
	Grana Harbour, Spain	M	43.4817	8.2600	25	HH, HI, HJ	DQ340209[b],DQ340210[b], DQ340211[b]
	Fornelos, Spain	N	43.4497	8.3103	18	HL, HM, HN	DQ340213[b], DQ340214[b], DQ340215[b]
	Ferrol, Spain	O	43.4792	8.2594	2	BS2	FJ528642[c], FJ528643[c]
	Roscoff, France	P	48.7267	3.9864	1	ST	AY116601[d]

			Mediterranean (MED)				
Botryllus schlosseri	Estartit Harbour, Spain	Q	42. 0542	3.2044	16	HA	DQ340205[b]
	Roses Harbour, Spain	R	42.2550	3.179722	11	HA, HE, HF	DQ340205[b], DQ340206[b], DQ340207[b]
	Canet Harbour, Spain	S	42.7042	3.0350	15	HA, HF, HG	DQ340205[b],DQ340207[b], DQ340208[b]
	Blanes Harbour, Spain	T	41.6750	2.7972	13	HA, HJ	DQ340205[b], DQ340211[b]
	Cubelles, Spain	U	41.1981	1.6661	14	HA, HK	DQ340205[b], DQ340212[b]
	Estaque Harbour, France	V	43.3603	5.3133	26	HA, HP	DQ340205[b], DQ340217*b*
	Vilanova, Spain	W	41.2103	1.7244	9	HT, HU	DQ340218[b], DQ340219[b]
	Ste. Marie La Mer, France	X	42.7233	3.0392	1	HV	DQ340221[b]
	Palamos, Spain	Y	41.8547	3.1433	2	HW	DQ340220[b]
	Tossa, Spain	Z	41.7214	2.9403	1	HA	FJ528641[e]

[a]This study
[b]Lopez-Legentil et al. (2006)
[c]Stach and Tuberville (2002)
[d]Johnson et al. (unpublished)
[e]Perez-Portela et al. (2009)

Table 3.2. Genetic diversity measures for the four ascidian species studied, indicating population, population sample size (n), number of haplotypes (Nh), number of polymorphic sites (ps), mean haplotype diversity (h) (standard deviation) and mean nucleotide diversity (p) (standard deviation). When the number of haplotypes = 1, ps, h and $p = 0$ by definition. NWA includes samples from Woods Hole, Massachusetts and Prince Edward Island (see Table 3.1).

Species	Population	n	Nh	ps	*h*	*p*
Botryllus schlosseri	Newfoundland	4	3	29	0.8330 (0.2224)	0.0302 (0.0205)
	NWA	4	1	0	---	---
Botrylloides violaceus	Newfoundland	3	1	0	---	---
	NWA	4	1	0	---	---
Halocynthia pyriformis	Newfoundland	5	2	2	0.4000(0.2373)	0.0012 (0.0012)
Boltenia echinata	Newfoundland	3	2	1	0.6667(0.3143)	0.0010 (0.0013)

Table 3.3. Sequence difference table (%) among the four ascidian species studied. Haplotype codes are cross-referenced in Table 3.1.

	Botrylloides violaceus	*Botryllus schlosseri*	*Halocynthia pyriformis*	*Boltenia echinata*
Botrylloides violaceus	0 (1 Haplotype)	17.7-29.5	25.6-25.8	23.6-23.7
Botryllus schlosseri		0.2-15.6	23.7-25.8	20.6-25.2
Halocynthia pyriformis			<1	22.7-25.1
Boltenia echinata				<1

Figure 3.1. Map of the Northwestern Atlantic indicating sample locations of *Botryllus schlosseri* (map courtesy of D. Deibel).

Figure 3.2. Map of the Northeastern Atlantic and the Mediterranean Sea indicating sample locations from GenBank of *Botryllus schlosseri*. (Johnson et al. (unpublished); Stach and Tuberville 2002; Lopez-Legentil et al. 2006; Perez-Portela et al. 2009) (map courtesy of D. Deibel).

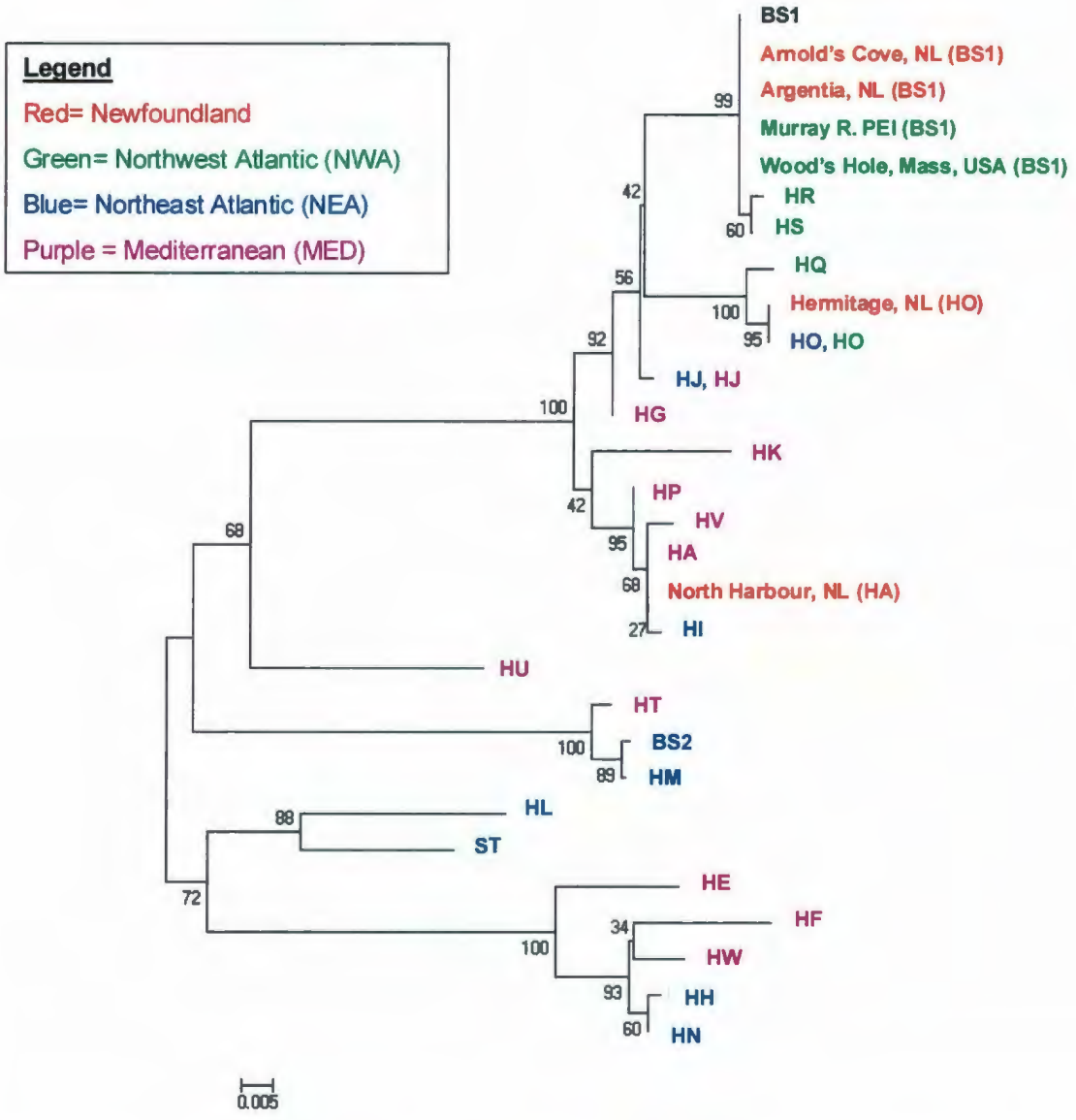

Figure 3.3. Neighbour-Joining tree for *Botryllus schlosseri* haplotypes using Tajima-Nei distance. Numbers at each branch indicates the percentage of times a node was supported in 1000 bootstrap replications. The scale bar indicates a distance equal to 0.005 nucleotide differences per site, equivalent to 2.6 nucleotide differences per partial sequence of 524 bp (i.e. 0.005 * 524). The haplotype codes correspond to the codes and locations from Table 3.1 and Fig. 3.2. The haplotypes from each region Newfoundland, the Northwestern Atlantic, the Northeastern Atlantic and the Mediterranean are color coded in the figure.

CHAPTER 4: SUMMARY

This thesis documents the first assessment of the presence of non-indigenous and potentially invasive species in high risk ports in Newfoundland. Ascidian tunicates have the potential to become invasive and spread to new areas via shipping vectors. Recently two non-indigenous ascidian species (*Botrylloides violaceus* and *Botryllus schlosseri*) have been discovered in several Newfoundland harbours. *Botryllus schlosseri* was found during a parallel rapid assessment survey in combination with my thesis research (Chapter 2), and *B. violaceus* was found during a subsequent survey in Belleoram harbour (Mckenzie and Deibel, unpubl.). Thus, invasive ascidians in Newfoundland will need to be managed in the future. In fact, the Government of Canada (DFO) has already conducted two extermination efforts at the wharf in Belleoram (2008 and 2009).

This study included the determination of the species composition of the benthic communities on wharf pilings in four Newfoundland harbours using several sampling methods (Chapter 2). This research can be used as baseline data for the determination of future invasions. The composition of these megabenthic communities was dominated mostly by *Mytilus* spp. This finding was expected as mussels are known to be competitive dominants and abundant members of fouling communities world wide (Mathieson et al. 1991; Greene and Grizzle 2007). However, the harbours varied in the diversity and abundance of organisms present. Argentia had highest species diversity, while Botwood was the least diverse but had the highest abundance of organisms (primarily *Mytilus* spp.).

The sampling methods varied in effectiveness of assessing fouling communities

(Chapter 2). Although *B. schlosseri* was found in Argentia, it was not detected with any of the sampling methods used. Therefore, it is important to continue to combine these sampling protocols with other protocols such the Rapid Assessment Surveys by SCUBA divers.

mtDNA partial COI sequence data was reported for two non-indigenous and two indigenous ascidian species in Newfoundland (Chapter 3). This anlysis was undertaken to determine whether these sequences can be used to distinguish among the species of Newfoundland ascidians. This information is required for future use of genetic markers for molecular identification of eggs and larvae of invasive Newfoundland ascidians. I also used the COI sequences to compare *B. schlosseri* Newfoundland haplotypes to haplotypes from GenBank from North America, the eastern Atlantic Ocean, and the Mediterranean Sea to confirm our taxonomic identification of the Newfoundland specimens and to construct a geneology to develop hypotheses concerning possible source populations. This geneology is required if vectors of invasion are to be identified and managed in the future.

Overall, haplotype diversity was very low for three of the four species, but much higher for the potentially invasive *Botryllus schlosseri*. There was less within-species variation in the COI sequence as opposed to between-species variation, which is necessary for the development of DNA-based tools for species identification of eggs and larvae for early detection of the presence of invaders. *B. schlosseri* was the only species for which sufficient COI data exists in GenBank to attempt a geneological assessment of potential source populations of Newfoundland specimens. Nearest neighbour analysis of genetic distance indicated that there may be three source populations seeding various

harbours in Placentia Bay, including from elsewhere in the Northwestern Atlantic Ocean (i.e. Prince Edward Island and Woods Hole, Massachusetts), as well as the Northeastern Atlantic Ocean (i.e., the Bay of Biscay), and the Mediterranean Sea (i.e., the Gulf of Valencia and the Gulf of Lion). There is sufficient evidence from this thesis to make inference about the potential source populations of *Botryllus schlosseri* in Newfoundland.

In the future, it will be important to continue surveys in Newfoundland harbours. These surveys should include more sites within and around Placentia Bay and Belleoram harbour, in order to determine if *Botryllus schlosseri* and *Botrylloides violaceus* populations are spreading to smaller bays and inlets via small boat traffic in the area. In conjunction with this monitoring, further samples should be collected for genetic analysis, in order to make a more complete assessment of within population variability in Newfoundland harbours and to narrow down the list of potential source populations. This strategy can help to implement management strategies in Newfoundland in order to prevent the spread of these invasive ascidians.

Appendices

Appendix 1. Pairwise sequence difference (%) among all the haplotypes of four ascidian species.

		Botrylloides violaceus	*Botrylus schlosseri*																			*Halocynthia pyriformis*		*Boltenia echinata*	
		BV1	BS1	BS2	HA	HE	HF	HI	HJ	HK	HL	HM	HN	HO	HP	HQ	HR	HS	HT	HU	HV	HW	ST	PH1	PH2	BE1	BE2
Botrylloides violaceus	BV1		20.6	21.2	19.7	20.4	21.4	19.8	20	20.2	18.5	21.2	20.8	20.2	19.5	20.6	20.6	20.8	21.2	17.7	19.8	20.2	18.3	25.6	25.8	23.6	23.7
Botrylus schlosseri	BS1			13.2	3.6	14.5	15.3	3.8	1.5	4.6	12.6	13.2	14.1	3.4	3.4	3.4	0.4	0.2	13.2	10.7	4	14.7	11.3	23.7	23.9	23.1	22.9
	BS2				12.2	13	14.3	12.4	12	12.8	12.8	0.2	12.8	13.5	12	13.9	13.5	13.4	1	10.9	12.6	12.4	11.6	23.9	24	22.9	22.7
	HA					13.2	14.7	0.2	4.3	2.9	11.1	12.2	12.8	4	0.2	4	4	3.8	12.2	9	0.4	13.4	10.7	23.7	23.9	21.6	21.4
	HE						4.6	13.4	13.4	14.7	9.9	12.8	3.8	14.9	13	14.9	14.9	14.7	12	10.7	13.5	3.6	10.1	24.4	24.2	21.6	20.6
	HF							14.9	14.5	15.5	11.8	14.1	2.7	15.6	14.5	15.6	15.3	15.5	13.4	12	15.1	2.9	12	24.4	24.2	21.9	21.8
	HI								2.7	3.1	11.3	12.4	13	4.2	0.4	4.2	4.2	4	12.4	9.2	0.6	13.5	11	23.7	23.9	21.6	21.4
	HJ									3.8	11.5	12	13	2.7	2.3	2.7	1.9	1.7	12	9.5	2.9	13.5	10.5	23.9	24	22.3	22.1
	HK										12.4	12.8	14.7	5	3.1	5	5	4.8	12.8	10.1	3.2	15.3	11.3	25.2	25.4	22.5	22.9
	HL											12.6	9.7	12.4	10.9	12.4	13	12.8	12.6	9.7	11.5	10.3	5.3	25	25	21.4	21.2
	HM												12.6	13.5	12	13.9	13.5	13.4	0.8	10.9	12.6	12.2	11.5	23.9	24	22.9	22.7
	HN													14.5	12.6	14.5	14.5	14.3	11.8	10.7	13.2	1.3	10.3	24.4	24.2	20.8	20.6
	HO														3.8	0.8	3.4	3.6	14.4	9.9	4.4	15.1	12	24.2	24.4	22.5	22.3
	HP															4	3.8	3.6	12	8.8	0.6	11.4	10.5	23.5	23.7	21.8	25.2
	HQ																3.1	3.1	13.9	10.3	4	15.1	12	24.6	24.8	22.9	22.7
	HR																	0.2	13.5	11.1	4	15.1	11.6	23.7	23.9	23.3	23.1
	HS																		11.5	10.9	3.8	14.9	11.5	23.9	24	23.4	23.1
	HT																			10.5	12.6	11.5	11.1	24	24.2	23.3	23.3
	HU																				9.4	10.7	9.7	24	24.2	23.4	21.2
	HV																					13.7	10.9	24	24.2	23.5	21.8
	HW																						10.9	23.8	23.6	21.9	20.8
	ST																							24.4	24.6	21	22.5
Halocynthia pyriformis	PH1																								0.3	22.7	25.1
	PH2																									25.2	25.1
Boltenia echinata	BE1																										0.1
	BE2																										